TRANSGENIC WHEAT AND NON-TARGET IMPACTS ON INSECT HERBIVORES AND FOOD WEBS

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TO CHRISTINE, MY FAMILY, MY FRIENDS
AND THE WIZARD OF OZ!

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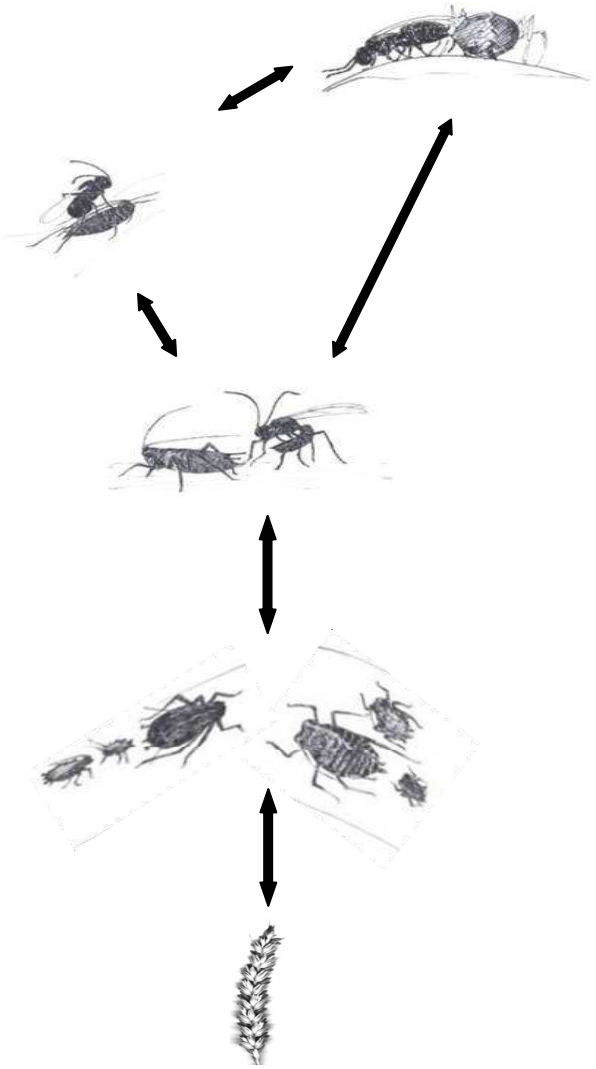

GENERAL INTRODUCTION

This PhD thesis combines research on ecological risks of genetically modified (GM) plants with plant-insect interactions and food web ecology. In this chapter I will review the current global state of GM crops, give a short overview over the concerns about ecological impacts of GM plants and introduce the study system. At the end of the chapter the goals of this thesis are stated.

1. Global state of transgenic crops

If not cited differently, all the numbers and facts about the global state of transgenic crops in this section come from the International Service for Acquisition of Agri-Biotech Applications (ISAAA) (James, 2009).

Since the first commercial release of a GM crop in 1996, the area grown with GM crops has increased from year to year and it is expected that this trend continues. In 2009 the estimated global area of GM crops was 134 million hectares. About 14 million farmers worldwide have adopted this new technology and have been growing biotech crops. And with the development of new, promising crops, adoption rates will rise further.

The four most important GM crops grown today are soybean, corn, cotton and canola. The most dominant GM trait is herbicide-tolerance and is deployed and grown on about 62 % of the global biotech crop area. The growth of area grown with GM crops that only possess insect-resistance by expressing Cry proteins derived from the soil bacterium *Bacillus thuringiensis* (Bt) has slowed down over the last few years and they now occupy around 16 %. Instead, stacked events containing herbicide-tolerance and insect-resistance genes are of growing importance and account for 21 % of all biotech crops grown. All these traits are so-called input traits and have the goal to reduce or substitute certain inputs in agricultural production systems (e.g. pesticides).

However, new GM plants are being developed continuously including plants with tolerance or resistance to other biotic and abiotic stresses (Sanvido *et al.*, 2007). During the past decade for example, a number of crop plants have been genetically engineered to enhance resistance to fungal diseases (Campbell *et al.*, 2002; Punja, 2001). Besides the expression of broad spectrum antimicrobial molecules (Anand *et al.*, 2003; Pasonen *et al.*, 2004), race-specific resistance genes have been found to be a potentially important resource for resistance breeding, not only for classical approaches but also for transgenic breeding (Feuillet *et al.*, 2003; Hulbert *et al.*, 2001).

2. Concerns about the ecological impacts of GM plants

Agricultural ecosystems inhabit numerous insect species which all are involved in complex interactions. Furthermore, as most ecological systems, agro-ecosystems are partly controlled and limited by bottom-up effects which transfer from the plant resource across herbivores (primary consumer) to the associated natural enemies of the herbivores (secondary consumers) (Hairston *et al.*, 1960; Hunter & Price, 1992; Oksanen *et al.*, 1981). Consequently, the identity, metabolism and genetics of a plant at the basis control the abundance and richness of consumer species and their interactions (Bukovinszky *et al.*, 2008; De Sassi *et al.*, 2006; Meister *et al.*, 2006; Omacini *et al.*, 2001). It is therefore not surprising that one of the main concerns associated to the release of GM plants is their potential to adversely affect non-target organisms. These include a range of arthropod species that fulfil important ecological functions such as herbivory, biological control, pollination and decomposition. Such effects on organisms that are not targeted by the introduced trait could occur either due to the expression of the trait (i.e. an insecticidal protein) or due to unintended, transformation-related effects.

Prior to the decision to cultivate a new GM crop variety and to avoid unacceptable harm to the environment the potential for adverse effects on non-target organisms is evaluated

as part of the environmental risk assessment (ERA) process (Conner *et al.*, 2003; Nap *et al.*, 2003; Romeis *et al.*, 2008) for which there are general guidelines and regulations in Switzerland (GTG), the European Union (EC 2001, 2002; EFSA 2006) and internationally (SCBD 2000).

3. Cereal aphids, parasitoids and their associations

The three most abundant cereal aphids that occur on wheat in Central Europe are the rose-grain aphid (*Metopolophium dirhodum*, Aphidinae: Macrosiphini), the bird cherry oat aphid (*Rhopalosiphum padi*, Aphidinae: Aphidini) and the grain aphid (*Sitobion avenae*, Aphidinae: Macrosiphini). They are all potential pests and vectors for plant viruses. They are attacked by a large community of natural enemies amongst which we find numerous parasitoid species that help keeping population sizes under control. Much is known about species identities for cereal aphids and their parasitoids and the quantification of their interactions is thus relatively simple.

Aphids are ideal biological model organisms to study plant-herbivore interactions for various reasons. They are small, have a short life-cycle, an asexual phase and hence can be cultivated as distinct clone lines which express variation in many different traits (Bieri *et al.*, 2009; Ferrari *et al.*, 2007; Ferrari *et al.*, 2008; Henter & Via, 1995; von Burg *et al.*, 2008; Vorburger, 2005). Because of their distinct life-cycle and their intimate connection with their host plants, aphids are widely used to investigate evolutionary as well as ecological questions about bottom-up effects of their host plants. Peccoud & Simon (2010) have recently reviewed the use of plant-aphid systems as an opportunity to assess divergent selection caused by host plant-preferences resulting in reproductive isolation also defined as ecological speciation.

Not only do aphids share a close relation with their host plants but also with their natural enemies especially parasitoids (Hymenoptera) (reviewed by Le Ralec *et al.*, 2010). Two groups of primary parasitoids have evolved to attack aphids: Aphidiinae

(Ichneumonoidea: Braconidae) and Aphelinidae (Chalcidoidea) (Powell, 1982). Females lay their eggs inside the living aphid and need to overcome potential immune responses by the host aphid. The fitness of the parasitoids that develop inside the aphids depends on the quality of the host (e.g. Nicol & Mackauer, 1999; Schadler *et al*., 2010).

Primary parasitoids themselves are attacked by secondary parasitoids of which two groups are being distinguished: hyperparasitoids (Figitidae: Charipinae: Alloxystini) and mummy parasitoids (from a number of different families of Hymenoptera, Parasitica). Hyperparasitoids attack the primary parasitoids before mummification. They delay their development until a mummy is formed by the primary parasitoid. Mummy parasitoids, however, do not have a delayed development and attack already formed mummies no matter whether they contain primary or hyperparasitoids. This means that mummy parasitoids feed on more than just one trophic level.

All this turns aphids and their parasitoids into the ideal miniature multitrophic model system to investigate bottom-up and top-down effects in food webs.

4. Cereal leaf beetles and chloropid gout fly

Besides aphids, there is a range of other pant-dwelling herbivores that attack wheat in Central Europe such as the cereal leaf beetle (*Oulema melanopus*, Coleoptera: Chrysomelidae) and the chloropid gout fly (*Chlorops pumilionis,* Diptera: Chloropidae). Both species belong to the most common insect herbivores on wheat in Switzerland and their feeding damage can eventually result in yield losses. Cereal leaf beetles and their larvae gnaw out longitudinal patches from the leaves resulting in typically white stripes on the leaves. Larvae of the chloropid gout fly feed on young plant tissues, including growing points which leads to stem deformation and growth stop.

5. The experimental wheat lines

The experiments in this study were conducted with GM spring wheat lines (*Triticum aestivum*) that were modified to express increased resistance against the fungal pathogen powdery mildew (*Blumeria graminis tritici* (DC.) Speer var. *tritici*). Powdery mildew of wheat is an obligate, biotrophic fungal pathogen that will infect all the green plant parts typically starting with the lowest leaves and then gradually infecting the rest of the plant forming a white, fluffy layer on the leave surface. It is widely distributed throughout the world and specially thrives in cool humid regions. The pathogen is easy to spot as the mycelium forms a white fluffy layer on the leaves and can, especially in wheat, also infect the ears (Börner, 1983). Infection with powdery mildew can eventually reduce yield (Oerke, 2006; Oerke & Dehne, 2004) and the overall loss potential of powdery mildew increases with the intensity of crop productivity (Oerke, 2006) which is a problem regarding the increasing intensification of agriculture.

We worked with two different types of GM wheat plants carrying two different types of resistance genes, i.e. alleles of the race-specific *Pm3* powdery mildew resistance gene or glucanase/chitinase genes from barley that should have a broad effect on all chitin containing fungi. The seeds of the experimental lines were provided by the Institute of Plant Biology, University of Zurich, and the Institute of Plant Science, ETH Zurich.

In total we worked with four independent *Pm3b*-transgenic plant lines and their corresponding, non-transgenic, segregant sister lines. These wheat lines are all based on the spring-wheat variety Bobwhite (SH98 26) that has no endogenous *Pm3b* gene and is generally sensitive to powdery mildew. The wheat lines were generated by biolistic transformation (Pellegrineschi *et al.*, 2002). The *Pm3b* gene was cloned from hexaploid wheat and expressed under the control of the maize ubiquitin promoter (Christensen & Quail, 1996). Transformants were selected on mannose containing media using the phosphomannose isomerase (PMI) coding gene as selectable marker (Reed *et al.*, 2001). After regeneration of

independent T0 transformants, four T1 segregants (offspring pairs) were selected for presence (transgenic lines *Pm3b* #1-4) or absence (control lines *S3b* #1-4) of the *Pm3b* transgene based on Southern hybridization (Southern, 2006). The transgenic lines contained one complete copy of the *Pm3b* (and an additional fragment in *Pm3b* #4) which segregated as a single Mendelian locus in the T1 generation. Homozygous lines of transgenic and control sister lines were then developed and multiplied.

The other plant type we worked with contained two independent transformation lines of the chitinase/glucanase-transgenic lines and one chitinase-transgenic line. They are based on the Swiss spring wheat variety Frisal, which is relatively tolerant to powdery mildew. Frisal lines were transformed introducing the β-1,3-glucanase coding sequence (HvGLU) (Leah *et al.*, 1991) and the seed chitinase coding sequence (HvCHI) (Leah *et al.*, 1991) both from barley. The two sequences were expressed under the rice actin1 promoter (McElroy *et al.*, 1990) and the maize ubiquitin (Christensen *et al.*, 1992) promoter respectively. Potential transgenic lines were identified through enzyme activity of the *Bar* gene in leaves (PAT-assay) and from a positive signal for chitinase, β-1, 3-glucanase and Bar probes in a Southern hybridization blot. Analysis for *Bar* expression identified lines with clear 3:1 segregation in the second generation, indicating a single transgene integration locus. For each line, seeds were produced from the sixth generation of transgenic lines.

It turned out that the transgenic Bobwhite plants (*Pm3b* #1-4) did indeed show a significantly increased resistance against powdery mildew whereas the transgenic Frisal lines (Chi/Glu (A13) & Chi (A9)) were as susceptible to the fungal pathogen as their non transformed counterpart.

6. Experimental field site and convertible glasshouse

The field site was located at the Agroscope Reckenholz-Tänikon Research Station (ART) in Zurich, Switzerland. Our field plots were part of a bigger field of the size of about one

hectare. The field was sown in spring as soon as conditions were suitable around the end of March and harvested after having dried out end of July. The wheat lines were grown in plots of 130 cm × 300 cm and the usual agricultural practices were applied except for insecticide applications.

The convertible glasshouse was also located at Agroscope ART about 300 m away from the field site. The roof and the side walls of the glasshouse automatically open under good weather conditions but close during unfavourable weather such as rainfall and heavy winds as well as during night. Even though it is defined as being a biologically contained system, the convertible glasshouse provides close to field conditions by exposing the wheat plants to outside environmental temperatures and allowing natural colonisation by insects and pathogens (Romeis *et al.*, 2007). Plants are grown in thermally insulated plastic containers (80 cm wide, 120 cm long and 80 cm high). The 20 containers are arranged in two rows. Each container is split into two plots each containing a separate central cylinder (26 cm diameter) resulting in 40 plots. The experimental wheat lines were grown in the central cylinder surrounded by buffer plants (i.e. plants of the same or the respective non-GM variety) simulating a near-field situation above ground. The sowing and harvest dates were similar to the ones in the field. Flowering ears were individually covered with pergamyn paper bags (Franz Grätzer & Co., Einsiedeln, Switzerland) and sealed to prevent pollen from escaping.

7. Goals and significance of the study

This thesis had five general goals: 1) to assess the direct and indirect impact of transgenic disease-resistant wheat plants on plant-dwelling insect herbivores, 2) to assess the impact of transgenic disease-resistant wheat plants on aphid-parasitoid food webs, 3) to elucidate the mechanisms by which transgenic disease-resistant wheat plants affect non-target organisms, 4) to assess the suitability of aphids as an indicator species and the applicability of food web

analyses for ERA, and 5) to assess the suitability of the convertible glasshouse system to generate data to support ERA.

In order to address these issues we performed field observations and conducted complementary experiments in the laboratory and glasshouse. By addressing basic ecological questions as well as taking a holistic food web approach this study adds to the knowledge about interactions between plants, herbivores and their antagonists. Moreover, this project serves as a case study for the assessment of potential benefits and risks for non-target organisms that could result from the use of GM crops in Switzerland.

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SUMMARY

This PhD work was conducted within the Wheat Cluster which is a subunit of the National Research Programme NRP 59 “Benefits and risks of the deliberate release of genetically modified plants”. The Wheat Cluster conducted field experiments with selected genetically modified (GM) spring wheat lines with enhanced resistance to powdery mildew. In this project we investigated the effects of the GM wheat plants on insect herbivores amongst which we worked with aphids and their associated food webs. We hypothesized that alterations in the metabolism of GM wheat plants could affect feeding behaviour, growth and fitness of insect herbivores and their natural enemies. We investigated our hypothesis in the field, in a semi-field environment (convertible glasshouse), and under confined conditions (glasshouse, climate chambers). The experiments in the field and the convertible glasshouse focussed on naturally occurring herbivore populations and on aphid-parasitoid food webs. Complementary experiments in the glasshouse and climate chambers were performed to better understand the mechanisms driving the plant-insect interactions.

In **Chapter 1** we present data from two years of field studies and two years of studies in the convertible glasshouse. In both systems we quantitatively counted the naturally occurring aphid population and collected parasitoid mummies for the construction of aphid parasitoid food webs. The aim of the study was to look if the genetic modification of the wheat lines affected parasitoid diversity and associated food web structures. In both years we found the three most common cereal aphid species *M. dirhodum, R. padi* and *S. avenae* and a total of 21 parasitoid species. Due to too few aphids and parasitoids present in the field, food web analysis was only conducted for the data collected in the convertible glasshouse. We found various significant effects of the different wheat lines on insect community structure up to the 4th trophic level. However, observed differences in the food web metrics were inconsistent between the two study years and there was as much variation between wheat

varieties as there was between GMO lines and their controls within varieties. Consequently observed effects are likely to be of little ecological relevance.

Chapter 2 describes a study where we looked at the impact of mildew-resistant wheat lines on different clone lines of the aphid *M. dirhodum*. The performance of 30 aphid clones on four different transgenic wheat lines and their four corresponding control lines was studied in a life-table experiment assessing a range of aphid life-history parameters. Looking at different aphid clones allowed us to assess whether impacts depended on aphid clone and whether there were aphid clone × wheat line interactions (genotype × environment). As expected we found significant clonal variation for all the measured life-history parameters. However, we did not find any major impact of the transgenic wheat lines on aphid performance except for a decrease of the total number of offspring by 3.33% produced on the GM lines compared to the control lines. There was no evidence for genotype × environment interactions. In sum, these results imply that there was no major difference in host plant quality of the GM lines compared to their control lines.

In **Chapter 3** we present the results of the insect monitoring including aphids, the chloropid gout fly and cereal leaf beetles. We assessed their abundance and the plant damage caused by them in the field and in the convertible glasshouse for two seasons. Besides the experimental wheat lines we also had a range of different commercially available wheat varieties as well as a Barley and a Triticale variety. We found that the transgenic Pm3b*#1* line had higher aphid populations compared to their control line. This was only found in the convertible glasshouse where aphid abundance and mildew infection were much higher compared to the field. It appeared that this effect was due to the decreased mildew infection of the resistant Pm3b*#1* lines and hence overall healthier plants. We provide further evidence for this in Chapter 4. We did not find differences between the GM wheat lines and the control lines for the other herbivores. Furthermore, the variation between the conventional wheat varieties and between the crops was much larger than between the experimental wheat lines.

The study presented in **Chapter 4** followed observations made in the field and the convertible glasshouse (Chapter 3) where we had seen that mildew infection seems to influence aphid performance: GM plants that had an increased resistance against powdery mildew and were thus less affected by the pathogen compared to their control lines carried larger aphid populations. To confirm this and learn more about the mechanism of this effect we conducted population experiments under controlled glasshouse conditions using the same experimental wheat lines and the two aphid species *M. dirhodum* and *R. padi*. We hypothesized that aphids feeding on infected plants will grow slower and will remain smaller compared to aphids on the non-infected plants. By combining three different, consecutive experiments we were able to clearly distinguish between variety effects, effects of the fungal pathogen and to exclude potential effects caused by the transformation process. We found that only *M. dirhodum* was affected by the presence of the fungal pathogen. In all three experiments we found smaller population sizes on infected plants whereas *R. padi* remained mostly unaffected by powdery mildew but was sensitive to wheat variety. We showed that *M. dirhodum* simply reacts to mildew infection and that there is no direct effect of the introduced transgene whatsoever.

ZUSAMMENFASSUNG

Diese Doktorarbeit wurde im Rahmen des Weizen-Konsortiums durchgeführt. Das Konsortium war Teil des Nationalen Forschungsprogram NFP 59 „Nutzen und Risiken der Freisetzung gentechnisch veränderter Pflanzen“. Das Weizen-Konsortium hat Feldexperimente mit verschiedenen ausgewählten, Mehltau-resistenten, gentechnisch veränderten (GV) Sommerweizen-Linien durchgeführt. In dieser Forschungsarbeit haben wir die Auswirkungen dieser GV Pflanzen auf pflanzenfressende Insekten und deren Antagonisten untersucht. Unter anderem haben wir uns mit Blattläusen und den mit ihnen assoziierten Nahrungsnetzen beschäftigt. Unsere Hypothese war, dass sich Veränderungen im Metabolismus der GV Pflanzen auf das Fressverhalten, das Wachstum und die Fitness von pflanzenfressenden Insekten und deren Feinden auswirken. Wir haben unsere Hypothese im Feld, einem halboffenen und einem konventionellen Glasshaus sowie in Klimakammern untersucht. Die Experimente im Feld und im halboffenen Glasshaus fokussierten sich auf natürlich vorkommende Herbivore und Blattlaus-Parasitoide Nahrungsnetze. Ergänzende Experimente im konventionellen Glasshaus und den Klimakammern wurden durchgeführt, um mehr über die Mechanismen zu erfahren, die die Pflanzen-Insekten-Interaktionen beeinträchtigen.

Im **Kapitel 1** präsentieren wir Daten von zwei Studienjahren im halboffenen Gewächshaus und im Feld. In beiden Umgebungen haben wir die natürlich vorkommenden Blattlauspopulationen quantitativ erfasst und Parasitoidenmumien gesammelt um die Blattlaus-Parasitoiden Nahrungsnetze zu konstruieren. Das Ziel der Studie war herauszufinden, ob sich die gentechnische Veränderung der Weizenpflanzen auf Parasitoidendiversität und die Struktur der Nahrungsnetze auswirkt. In beiden Jahren fanden wir die drei häufigsten Getreideblattläuse *M. dirhodum*, *R. padi* und *S. avenae* sowie insgesamt 21 Parasitoidenarten. Aufgrund zu niedriger Blattlaus- und Mumiendichten im Feld

konnte die Analyse der Nahrungsnetze nur für die Daten aus dem halboffenen Gewächshaus gemacht werden. Wir fanden diverse Effekte der verschiedenen Weizenlinien auf die Insektengemeinschaft bis hinauf zur vierten trophischen Ebene. Jedoch waren diese Unterschiede inkonsistent zwischen den Studienjahren. Des Weiteren fanden wir gleich viel Variation zwischen den Weizen-Sorten wie zwischen den GV-Linien und den Kontrollinien innerhalb einer Sorte. Deshalb sind die beobachteten Unterschiede höchst wahrscheinlich von untergeordneter, ökologischer Relevanz.

Kaptiel 2 beschreibt eine Studie in der wir die Auswirkungen von Mehltau-resistentem GV Weizen auf 30 verschiedene Klonlinien der Blattlaus *M. dirhodum* untersuchten. Die Blattlausklonlinien wurden auf vier unterschiedlichen GV Weizenlinien und deren dazugehörigen Kontrollinien gehalten und wir haben eine Reihe von Lebenstafel-Parameter untersucht. Indem wir unterschiedliche Klonlinien untersuchten, konnten wir unterscheiden, welche Effekte durch den Blattlausgenotyp bestimmt wurden und ob es Klonlinien × Weizenlinien Interaktionen gab (Genotyp × Umwelt). Wie erwartet, fanden wir signifikante klonale Variation für alle Lebenstafel-Parameter. Wir fanden jedoch keinen grossen Einfluss der GV Weizenlinien auf die Biologie der Blattläuse mit Ausnahme von einer um 3.33 % reduzierten Anzahl produzierter Nachkommen auf den GV Weizenlinien im Vergleich mit den Kontrollinien. Es gab keinen Nachweis für Genotyp × Umwelt-Interaktionen. Zusammenfassend zeigen diese Resultate, dass sich Nahrungsqualität der GV Wirtspflanzen verglichen mit den nicht GV Pflanzen für Blattläuse nicht entscheidend geändert hat.

In **Kapitel 3** präsentieren wir Resultate vom Insektenmonitoring im Feld und im halboffenen Gewächshaus. Während zwei Jahren wurden Vorkommen von Blattläusen, der Gelben Getreidehalmfliege und dem Getreidehähnchen sowie der Schaden der letzteren beiden erfasst. Neben den experimentellen GV Weizenpflanzen hatten wir auch eine Reihe kommerziell erhältlicher Weizensorten sowie eine Gerste- und eine Triticalesorte im Versuch.

Im Gewächshaus, wo die Blattlausdichte und Mehltauinfektion höher war als im Feld, fanden wir mehr Blattläuse auf der transgenen Pm3b*#1* Linie verglichen mit deren Kontrollinie. Dies war vermutlich eine Folge des tieferen Mehltau-Befalls der resistenten Pm3b*#1* Pflanzen und deren damit einhergehenden besseren Gesundheitszustandes. Dafür erbringen wir in Kapitel 4 weitere Beweise. Für die anderen Herbivoren fanden wir keine Unterschiede zwischen den GV Pflanzen und ihren Kontrollen. Des Weiteren waren die Unterschiede zwischen den kommerziellen Sorten grösser, als zwischen den experimentellen Pflanzen.

In **Kapitel 4** sind wir der im Feld und im halboffenen Glasshaus gemachten Beobachtung nachgegangen, dass GV Pflanzen mit tieferen Mehltau-Infektionen mehr Blattläuse zu beherbergen scheinen (Kapitel 3). Um diesen Zusammenhang zu bestätigen, haben wir im Glasshaus unter kontrollierten Bedingungen Populationsexperimente mit denselben Weizenlinien und den zwei Blattlausarten *M. dirhodum* und *R. padi* durchgeführt. Unsere Hypothese war, dass Blattläuse, die auf von Mehltau-befallenen Pflanzen fressen, langsamer wachsen und kleiner bleiben, im Vergleich zu Blattläusen, die auf gesunden Pflanzen fressen. Indem wir drei aufeinandefolgende Experimente durchführten, konnten wir klar zwischen Sorteneffekten und Mehltau-Effekten unterscheiden sowie potentielle GV-Effekte auschliessen. *Metopolophium dirhodum* war in allen drei Experimenten von der Anwesenheit des Pilzes betroffen und zeigte kleinere Populationsgrössen auf den infizierten Pflanzen. *Rhopalosiphum padi* reagierte grösstenteils nicht auf Mehltau, zeigte jedoch eine Sortenabhängikeit. Wir zeigten, dass *M. dirhodum* nur auf die Anwesenheit von Mehltau reagierte und dass die GV-Weizenlinien keinen direkten Einfluss haben.

CHAPTER 1

Structure of an aphid-parasitoid community on transgenic disease- resistant wheat

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Aphid-parasitoid community structure on genetically modified wheat

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Short title: Aphid-parasitoid food webs on GM wheat

SUMMARY

Since the introduction of genetically modified (GM) plants, one of the main concerns has been their potential effect on non-target insects. Many studies have looked at GM plant effects on single non-target herbivore species or on simple herbivore-natural enemy food chains. Agro-ecosystems, however, are characterized by numerous insect species which are involved in complex interactions, forming food webs. In this study we looked at transgenic disease-resistant wheat (*Triticum aestivum*) and its effect on aphid-parasitoid food webs. We hypothesized that the genetic modification of the wheat lines directly or indirectly affect aphids and that these effects cascade up to change the structure of the associated food webs. Over two years, we studied different experimental wheat lines under semi-field conditions. We constructed quantitative food webs to compare their properties on GM lines with the properties on corresponding non-transgenic controls. We found significant effects of the different wheat lines on insect community structure up to the 4^{th} trophic level. However, the observed effects were inconsistent between study years and the variation between wheat varieties was as big as between GM plants and their controls. This suggests that impact of GM wheat on food web structure may be negligible and potential ecological effects on non-target insects limited.

Keywords: insect food web, powdery mildew, non-target effect, community genetics

1. INTRODUCTION

Insect species that inhabit agricultural ecosystems are involved in complex interactions, forming so-called food webs [1]. The diversity and complexity of such food webs are considered to be important factors which determine ecosystem function and stability. Insect host-parasitoid systems are influenced by plant traits, which can lead to large effects on food web structure [2]. Due to their biology, interactions between aphids, primary parasitoids and secondary parasitoids can easily be quantified and have proven to be a useful system for exploring multi-trophic interactions.

Genetic modification (GM) of plants can cause differences between the transformed varieties and their conventional counterpart. For instance, changes in phloem sap composition can affect organisms that feed upon these plants including sap-feeding aphids [3]. In an aphid-parasitoid-hyperparasitoid food web, the effects of host plant traits can cascade up as far as the 4^{th} trophic level [2], even when there are no effects on the herbivores [4].

We hypothesized that alterations on the plant level due to genetic modification could affect the associated aphid-parasitoid-hyperparasitoid food web. We studied this on two disease-resistant GM wheat lines (*Triticum aestivum*), their respective non-transformed controls and a conventional line.

2. MATERIAL AND METHODS

(a) *Study organisms*

Metopolophium dirhodum, *Rhopalosiphum padi* and *Sitobion avenae* are common cereal aphids in Europe [5]. They are attacked by primary parasitoids that oviposit inside the living aphid. The parasitoid larva pupates inside the dead aphid, forming a “mummy”. Primary parasitoids are attacked by two guilds of secondary parasitoids i.e. hyperparasitoids and mummy parasitoids. Hyperparasitoids attack primary parasitoids before mummification,

whereas the more generalist mummy parasitoids attack the already mummified aphid irrespective of whether it contains a primary parasitoid or a hyperparasitoid.

(b) *Plant material*

We used five wheat lines belonging to three different spring-wheat varieties: Bobwhite, Frisal and Rubli. For Bobwhite and Frisal, we each had a transgenic line and its corresponding control line. The transgenic line of Bobwhite (Pm3b*#1*) carries the transgene *Pm3b* of hexaploid wheat, which confers race-specific resistance to wheat powdery mildew [6]. The null-segregant line Sb*#1* was used as corresponding control line. The second wheat pair consisted of the non-transformed variety Frisal as a control and the GM Frisal line Chi/Glu(A13) containing the anti-fungal barley seed chitinase and β-1, 3-glucanase [7, 8]. This line, however, has not shown to display enhanced resistance to powdery mildew [8]. The variety Rubli is commercially grown in Switzerland.

(c) *The convertible glasshouse*

The experiments were carried out in a convertible glasshouse that approximates field conditions by exposing the plants to outside environmental temperatures and allowing natural colonisation by insects and pathogens. The roof and side walls automatically open under good weather conditions but close during strong winds and rainfall and during the night [9]. Plants were grown in 40 plots (80 cm × 60 cm × 80 cm) arranged in two rows, each plot containing a separate central cylinder (26 cm diameter) in which ten experimental wheat plants were grown. Buffer plants (i.e. non-transformed plants of the same variety) were grown around the central cylinder simulating a near-field situation. Each wheat line was replicated eight times in blocks of five adjacent plots in a row containing the five wheat lines in randomized order. Plants were sown end of March and harvested end of July. Before sowing, basic fertilizer was added to the soil (per plot: 2008: 5.8g P, 7.2g K, 1.7g Mg, 8.7g N; 2009: 10.32g P, 8g K, 9g

$Mg(NO_3)_2$). Plants were watered as required. Flowering ears of all wheat lines were covered with pergamyn paper bags to prevent pollen from escaping. No pesticides were applied.

(d) *Sampling*

Experiments were conducted in 2008 and 2009. Every week, we counted and identified all aphids and collected all mummies on the experimental plants in the central cylinder from mid May until harvest. Parasitoid mummies were stored separately in gelatine capsules at room temperature. After four months, emerged parasitoids were identified to species level using the same keys as Müller *et al.* 1999 [10].

(e) *Quantitative food web metrics & data analysis*

Traditional binary measures of food web structure are highly sensitive to sample size [11]. Therefore, we used measures based on Shannon information theory [12], as described in Bersier et al. [13] and adapted for host-parasitoid webs [2], which use the densities of species and the frequency of interactions: i) parasitoid diversity, which is equal to species richness when all species are equally abundant but takes on smaller values when abundances are uneven; ii) link evenness, which equals one when all trophic links have equal frequencies and asymptotically approaches zero for highly uneven frequencies; iii) quantitative realised connectance, which is the observed link diversity as a proportion of maximum possible link diversity and is a measure of the complexity of the network. Since secondary parasitoids cannot be unambiguously linked to primary parasitoids [10], we calculated all the food web metrics separately for the aphid-primary parasitoid and the aphid-secondary parasitoid matrices.

We used linear models (LM) to analyse all the food web metrics. We conducted analyses across both years and for each year separately. Parasitoid diversity was square root transformed; link evenness and realised connectance were arcsine transformed. Further we

analysed cumulative aphid and mummy abundance, parasitoid hatching success and parasitism rates. Aphid and mummy abundance were square-root transformed, parasitism was arcsine transformed and both analysed using LM. Hatching success was analysed with a generalized linear model with binomial errors. All analyses were done with the statistical software R (R development core team).

3. RESULTS

Three cereal aphid species were recorded in the two study years: *M. dirhodum* (mean seasonal density per cylinder: 2008: 88.6; 2009: 248.4), *R. padi* (2008: 66.3; 2009: 76.6) and *S. avenae* (2008: 2.4; 2009: 17.2). Overall aphid abundance was significantly higher in 2009 ($F_{1,66}$ = 160.71, $p < 0.001$) and depended on variety ($F_{2,66}$ = 5.26, $p = 0.007$) and on the variety × GM interaction ($F_{1,66}$ = 4.08, $p = 0.047$) which was caused by a higher total aphid abundance on Pm3b*#1* compared to its control line Sb*#1*, whereas abundance was the same on Chi/Glu(A13) and Frisal. The number of mummies was significantly higher in 2009 compared to 2008 ($F_{1,66}$ = 203.23, $p < 0.001$) and was positively aphid-density dependent ($p < 0.001$, $R = 0.888$). Parasitism rate was low at 2% in both years and negatively correlated with aphid density ($p = 0.023$, $R = -0.253$). Neither wheat variety nor GM or their interaction had an effect on parasitism (variety: $F_{2,66}$ = 2.10, $p = 0.130$; GM: $F_{1,66}$ = 2.70, $p = 0.105$; variety × GM: $F_{1,66}$ = 2.04, $p = 0.158$).

From a total of 3492 mummies collected in both years, 2108 hatched (60.6 %). Hatching success was influenced by host aphid ($p < 0.001$; *M. dirhodum* > *R. padi* > *S. avenae*) and by year ($p < 0.001$; 2009 > 2008). We identified 21 parasitoid species, of which eight were primary parasitoid species, seven hyperparasitoid species and six species were mummy parasitoids (Table 1). In a parallel field study using the same wheat lines we found the same aphid and parasitoid species (Appendix I).

The analysis across both years revealed significant annual variation for all the food web metrics except realised connectance of secondary parasitoids (Fig. 1 & 2). The variety × GMO interaction was significant for link evenness of primary parasitoids ($F_{1,66}$ = 8.312, p = 0.005). No other differences were found.

Separate analysis of the two years revealed several different, inconsistent effects. In 2009, we only found significant effects on the primary parasitoid web. Primary parasitoid diversity significantly differed between the three varieties (Fig. 1b) and was highest on Rubli. The variety × GM interaction was significant for primary parasitoid link evenness (Fig. 1d). In contrast, in 2008 we only found effects on the secondary parasitoid web: a significant variety effect on link evenness (Fig. 2c) was found and significantly higher realised connectance on the GM plant lines (Fig. 2e). The aphid-parasitoid food webs are shown in the Appendix II.

4. DISCUSSION

While other studies have assessed the effects of GM plants on aphids and their parasitoids [14, 15], this is the first one to look at quantitative food web metrics of an aphid-parasitoid community.

Aphid abundance on the GM line Pm3b*#1* was higher compared to the control line Sb*#1*. This could directly be due to genetic differences in the plants or to indirect effects caused by the difference in mildew infection. As expected, the Pm3b*#1* plants showed enhanced powdery mildew-resistance compared to the control Sb*#1* whereas Chi/Glu(A13) was equally susceptible to powdery mildew as its control line Frisal. In an earlier study we showed that the Pm3b*#1* plants did not affect the performance of *M. dirhodum* in the absence of powdery mildew [16]. We therefore suspect that an indirect effect through mildew causes this difference in aphid abundance.

Parasitism rate was negatively host density-dependent, indicating that parasitoids keep up with aphid population growth only to a certain point. The early presence of parasitoids in the

field seems therefore crucial for keeping aphid populations under control as has been suggested before [17]. Parasitoid diversity remained generally unaffected by the wheat lines except for primary parasitoid diversity in 2009 which was higher on Rubli. Higher parasitoid diversity has been linked to higher parasitism rates [18], an effect that was not observed here. Link evenness was affected in the primary parasitoid and in the secondary parasitoid webs but differently so. Variety affected the secondary web in 2008, whereas GM within the varieties affected link evenness of the primary parasitoid web in different directions in 2009. Food web structure on the transgenic Pm3b*#1* line was more dominated by a few stronger links, whereas on the control line Sb*#1* they were more evenly distributed. For the GM line Chi/Glu(A13) and its control, this was the other way around. In 2008, connectance of secondary parasitoid food webs was increased on the GM lines compared to the control lines with potential implications for food web stability [19].

Quantitative food webs allow to compare the diversity and complexity of species assemblages which are considered to be major factors determining ecosystem functioning and stability. Despite the observed changes in community structure among the different wheat lines studied, it is difficult to draw conclusions about the ecological implications of these effects. Observed differences in community structure were inconsistent between the two study years, and there was as much variation between wheat varieties as there was between GM and control lines within varieties. This phenomenon has been observed before in other studies dealing with non-target effects of GM crops [20]. Plant characteristics seem to be more distinct between varieties than between GM plants and the corresponding controls. Therefore, we believe that the observed GM effects are of little ecological significance and fall within the natural variation observed among cultivars.

Mario Waldburger helped with the sampling, Beat Keller and Christof Sautter provided the plant material. Food web graphs were drawn using a Mathematica code provided by H.C.J

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Table 1: Aphid-parasitoid associations across two years with respective food web codes in brackets as they appear in Figure 1 & 2 of Appendix II. Numbers in the table correspond to the individuals found for the specific aphid-parasitoid association.

	Metopolophium dirhodum (21)	*Rhopalosiphum padi* (22)	*Sitobion avenae* (23)
PRIMARY PARASITOIDS			
Aphelinus asychis (31)		1	
Aphelinus varipes (32)		60	
Aphidius ervi (33)	45	7	15
Aphidius picipes (34)	35	11	7
Aphidius rhopalosiphi (35)	479	142	10
Binodoxys sp. (38)		2	
Ephedrus plagiator (36)	7	20	3
Praon volucre (37)	309	13	7
SECONDARY PARASITOIDS			
Hyperparasitoids			
Alloxysta brachyptera (47)		1	
Alloxysta brevis (48)		2	
Alloxysta f-sp. (41)	1	3	
Alloxysta hepaptoma (42)		1	
Alloxysta r-sp. (43)	4	47	1
Alloxysta victrix (45)	136	12	4
Phaenoglyphis villosa (46)	77	16	4
Mummy parasitoids			
Asaphes suspensus (51)	66	26	6
Asaphes vulgaris (52)	290	54	6
Coruna clavata (53)	98	2	3
Dendrocerus carpenteri (54)	18	1	2
Dendrocerus laticeps (55)		2	
Pachyneuron sp. (56)	34	5	13

FIGURE 1

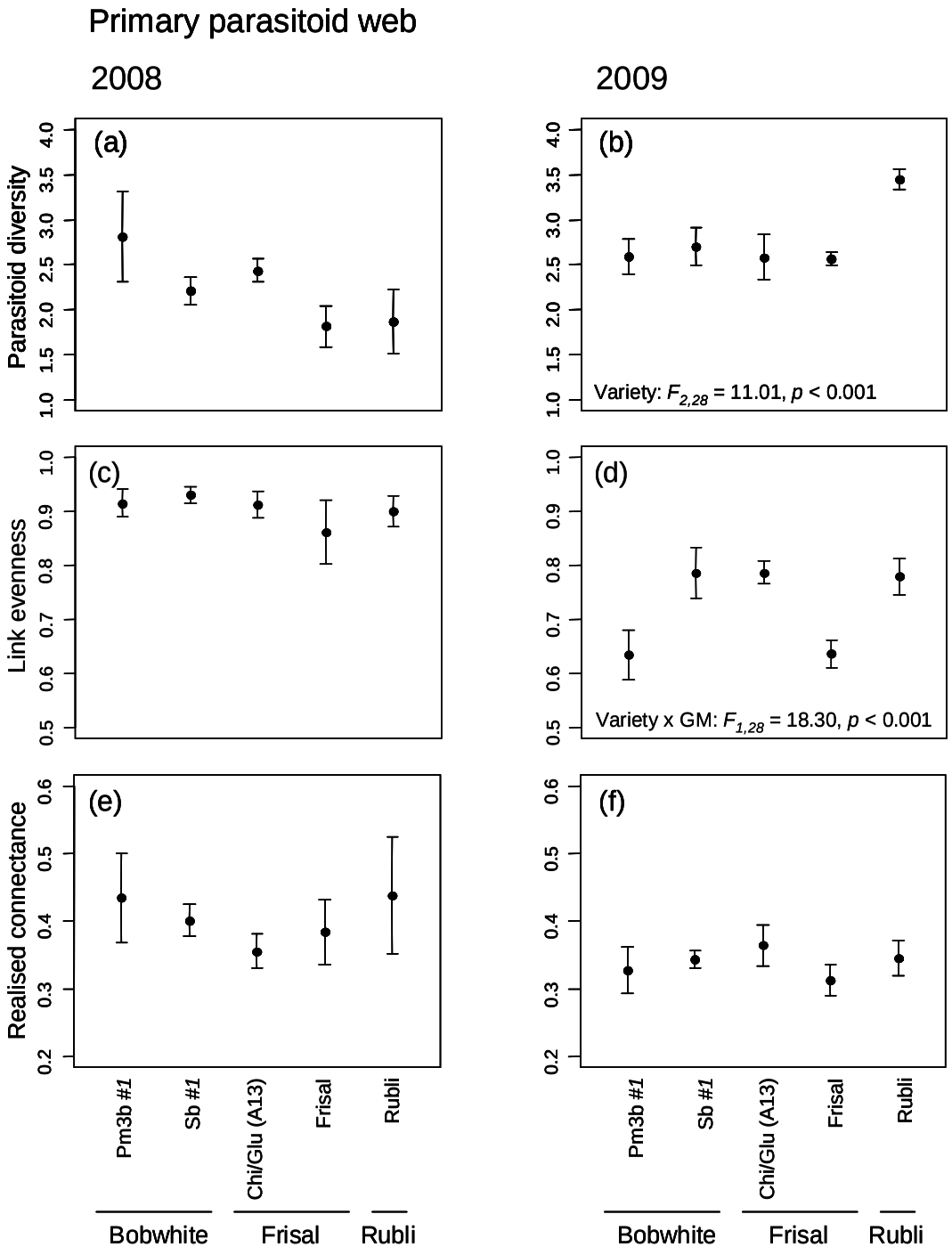

Figure 1. Means (± SEM) of the calculated food web metrics of the primary parasitoid web per wheat line and year. (a) & (b) Parasitoid diversity, (c) & (d) Link evenness, (e) & (f) Realised connectance.

FIGURE 2

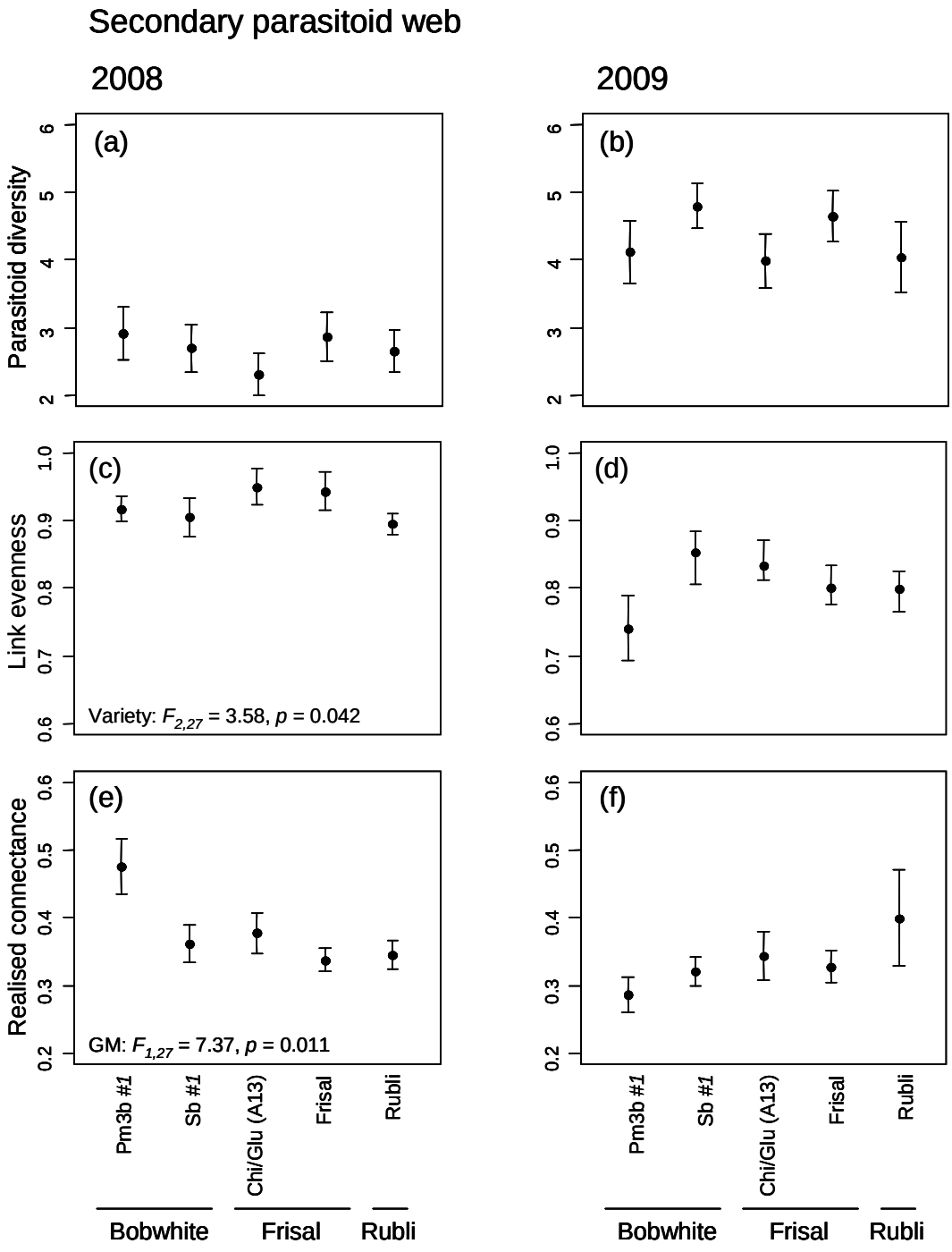

Figure 2. Means (± SEM) of the calculated food web metrics of the secondary parasitoid web per wheat line and year. (a) & (b) Parasitoid diversity, (c) & (d) Link evenness, (e) & (f) Realised connectance.

APPENDIX I

Field trial

An experiment with the same wheat lines was conducted in a parallel field study located at the Agroscope Reckenholz-Tänikon Research Station (ART) in Zurich, Switzerland. The research station is located just outside the city of Zurich in a rural area surrounded by fields, forests and orchards. The field site was only about 300 meters apart from the convertible glasshouse. Our field plots were part of a bigger field of the size of about one hectare. The field was sown during the last week of March as soon as conditions were suitable and harvested after having dried out by the end of July. The wheat lines were grown in plots of 130cm × 300 cm. Each line was replicated four times in 2008 and five times in 2009. The replicates were randomly assigned to spatial blocks. We bimonthly sampled 20 randomly chosen tillers per plot and counted all the present aphids and collected the parasitoid mummies. However, aphid and parasitoid numbers were too low in both years to use the data for statistical analyses therefore we present here a merely descriptive species list. We found the same three cereal aphid species as in the glasshouse (*Metopolophium dirhodum, Rhopalosiphum padi, Sitobion avenae*). We were only able to identify 201 parasitoid individuals belonging to 13 different species which are summarized in Table 1. Due to the small number of aphids and mummies present in the field we probably under-sampled the real number of parasitoid species present. However, the aphid and parasitoid species we did find were the same as in the convertible glasshouse where protection against unfavourable weather conditions seems to boost natural aphid and parasitoid population growth which might actually be an advantage when studying the effects of GM wheat on insect herbivores.

Table 1: Summarized aphid-parasitoid associations recorded in the field for both years (2008, 2009). The three cereal aphid species are listed across the top. The identified parasitoid species are listed below with primary parasitoids being listed first, followed by secondary parasitoids (i.e. hyperparasitoids and mummy parasitoids). The numbers in the table are the number of individuals found for the specific aphid-parasitoid association.

	Metopolophium dirhodum	*Rhopalosiphum padi*	*Sitobion avenae*
PRIMARY PARASITOIDS			
Aphelinus asychis			1
Aphidius ervi	2	4	
Aphidius picipes	14	1	
Aphidius rhopalosiphi	64	17	2
Ephedrus plagiator	10	9	3
Praon volucre	1		
SECONDARY PARASITOIDS			
Hyperparasitoids			
Alloxysta r-sp.	7	1	
Alloxysta victrix	3		
Phaenoglyphis villosa		1	
Mummy parasitoids			
Asaphes suspensus	11	9	7
Asaphes vulgaris	4	1	
Coruna clavata	1		
Dendrocerus carpenteri	22	2	4

APPENDIX II

Food web graphs

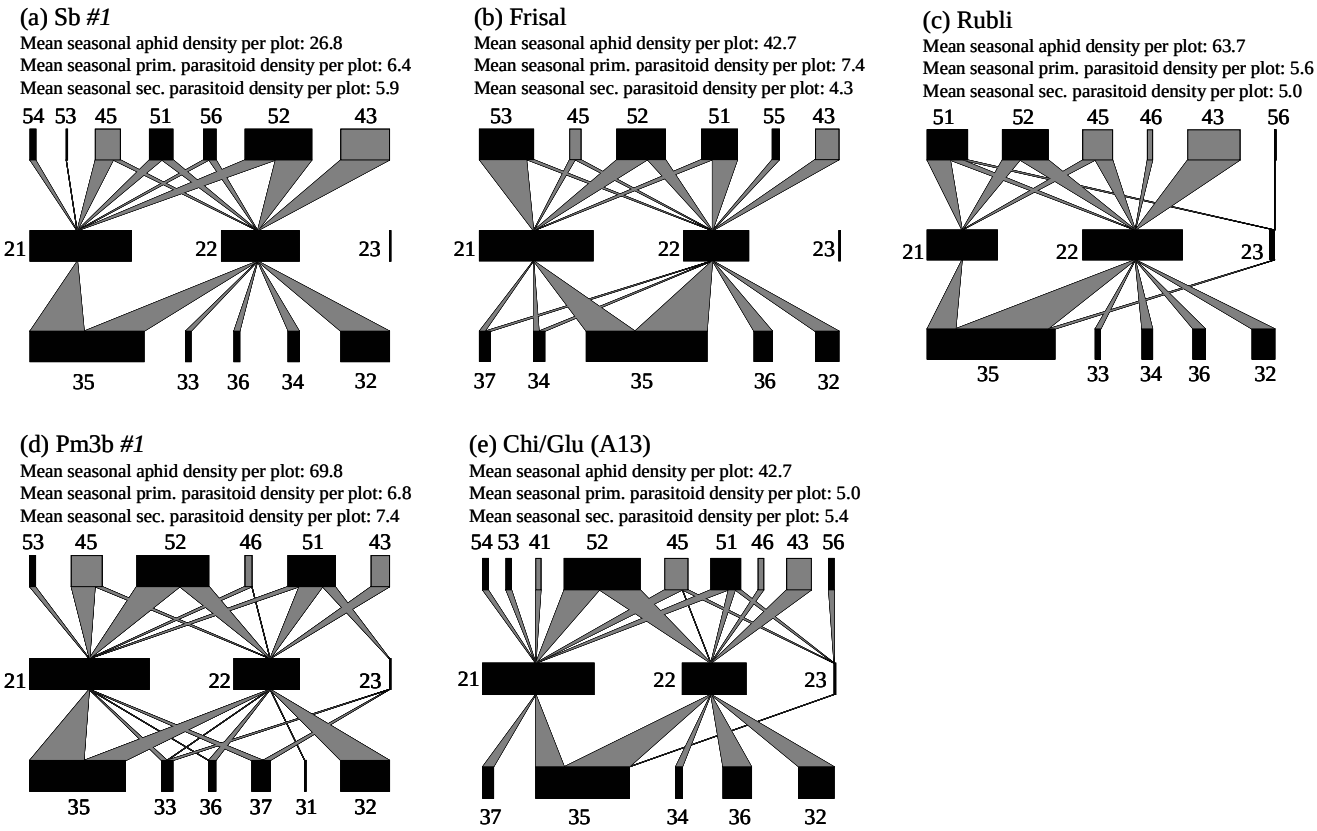

Figure 1: Aphid-parasitoid interaction webs on the five wheat lines in 2008 summed over the eight replicates. (a) Sb*#1* (control line 1), (b) Frisal (control line 2), (c) Rubli (conventional line), (d) Pm3b*#1* (GM line 1), (e) Chi/Glu(A13) (GM line2). The food web graphs were drawn with Mathematica and the bars in the middle represent the aphid species. Parasitoids were arranged below (primary parasitoids) and above (secondary parasitoids with hyperparasitoids in grey and mummy parasitoids in black). The width of the bars corresponds to relative abundances per plot. The species codes are given in Table 1 of the main text.

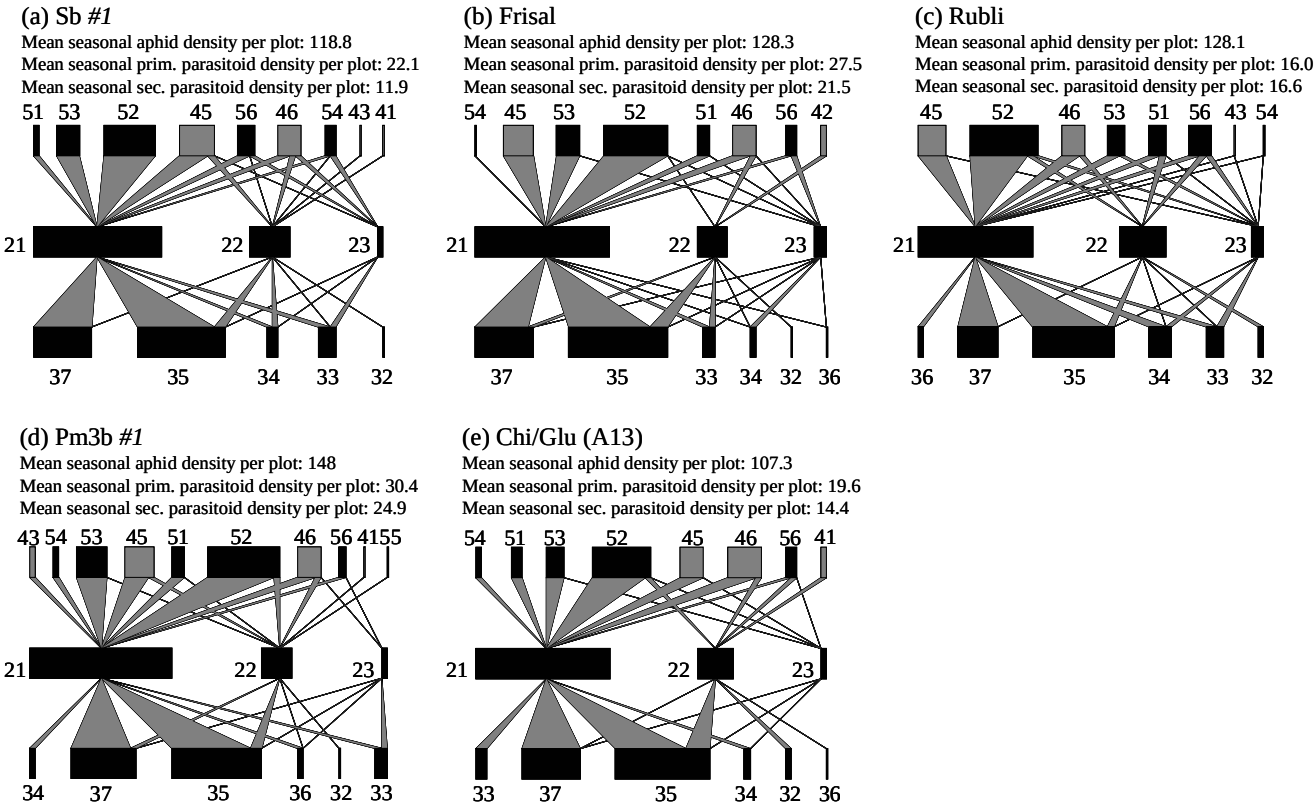

Figure 2: Aphid-parasitoid interaction webs in 2009. The webs are read and interpreted as in Figure 1 of the Appendix II. (a) Sb *#1*, (b) Frisal, (c) Rubli, (d) Pm3b *#1*, (e) Chi/Glu (A13).

CHAPTER 2

Transgenic disease-resistant wheat does not affect the clonal performance of the aphid *Metopolophium dirhodum* Walker

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Transgenic disease-resistant wheat does not affect the clonal performance of the aphid *Metopolophium dirhodum* Walker

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Abstract

Ever since the introduction of transgenic crops one of the main concerns has been their potential impact on non-target organisms. In this study we looked at the impact of transgenic disease-resistant wheat on different clones of the aphid *Metopolophium dirhodum*. Looking at different clones allowed us to assess whether impacts depended on aphid clone and whether there were aphid clone × wheat line interactions (genotype × environment interactions). The performance of 30 aphid clones on four different transgenic wheat lines and their corresponding control lines was studied in a life-table experiment assessing the following aphid life-history parameters: development time, adult weight, daily fecundity, total offspring number and the fitness estimate F_l'. As expected, we found significant variation among aphid clones for all the measured life-history parameters. However, our experiments did not reveal any major impact of the transgenic wheat lines on aphid performance. The only significant difference was found for total offspring number which was reduced by 3.33% on the transgenic wheat lines compared with the control lines. There was no evidence for a genotype × environment interaction between aphid clones and wheat lines. In sum, our results imply that the genetically modified plants used in this assay were of similar host plant quality as the non-transformed control lines and that the introduced transgene had no major effect on the performance of individual aphid clones.
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Zusammenfassung

Eines der Hauptinteressen seit der Einführung transgener Nutzpflanzen gilt deren möglicherweise negativen Einfluss auf Nicht-Zielorganismen. In dieser Studie untersuchten wir die Auswirkung von Krankheits-resistentem Weizen auf verschiedene Klone der Blattlausart *Metopolophium dirhodum*. Indem wir verschiedene Klone untersuchten, konnten wir testen, ob zwischen Blattläusen und Weizenlinien Genotyp × Umwelt-Interaktionen vorkommen. In einem "Lebenstafel-Experiment" verglichen wir die demographischen Parameter (Entwicklungsdauer, Adultgewicht, durchschnittliche Fekundität pro Tag, totale Anzahl Nachkommen, Fitnessparameter F_l') von 30 Blattlausklonen auf vier transgenen Weizenlinien und deren dazugehörigen Kontrolllinien. Wie erwartet, unterschieden sich die Blattlausklone genetisch voneinander in allen gemessenen Parametern. Die Blattläuse auf transgenen Weizenlinien unterschieden sich jedoch praktisch nicht von jenen auf den Kontrollinien. Der einzige signifikante Unterschied war

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eine um 3.33% reduzierte Gesammtzahl der Nachkommen auf den transgenen Weizenlinien verglichen mit den dazugehörigen Kontrolllinien. Wir fanden keine Genotyp × Umwelt-Interaktionen zwischen Blattlausklonen und Weizenlinien. Diese Resultate zeigen, dass die in unserem Experiment verwendeten Weizenlinien mit oder ohne Transgen sich hinsichtlich ihrer Wirtsqualität für *M. dirhodum* kaum unterscheiden und dass auch die verschiedenen Blattlausklone keine unterschiedlichen Reaktionen auf die Weizenlinien zeigen.
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Keywords: Non-target organism; Insect herbivore; Genotype × environment interaction; Bottom-up effects; Powdery mildew

Introduction

The increasing use of genetically modified (GM) crops has raised concerns about their potential detrimental effects on the environment. Since the identity, metabolism and genetics of a plant affect the abundance and richness of its consumer species, one of the main ecological concerns regarding the release of GM crops is their impact on organisms which are not the target of the introduced transgene, the so-called non-target organisms (Conner, Glare, & Nap, 2003; Dale, Clarke, & Fontes, 2002; Sanvido, Romeis, & Bigler, 2007). Supposedly, plant-dwelling insect herbivores such as aphids are the species that are most exposed to potential changes in plant quality due to genetic modification.

Aphids belong to the world's major agricultural pests and react sensitively to metabolic and physiological changes in their host plants. A range of studies showed the crucial role of not only phloem sap composition (Karley, Douglas, & Parker, 2002; Kazemi & Vanemden, 1992; Sandstrom & Pettersson, 1994; Weibull, 1987) on aphid performance but also of secondary plant metabolites (Givovich, Sandstrom, Niemeyer, & Pettersson, 1994; Leszczynski, Tjallingii, Dixon, & Swiderski, 1995; Niemeyer, 1988; Niraz, Leszczynski, Ciepiela, & Urbanska, 1985). Genetic modification can alter the physiology and metabolism of plants as it has been reported in studies using transgenic insect-resistant maize (Escher, Käch, & Nentwig, 2000; Saxena & Stotzky, 2001; Zurbrügg, Höhnemann, Meissle, Romeis, & Nentwig, 2010). Furthermore, genetic modification can influence phloem sap composition resulting in a changed aphid performance (Hunt et al., 2006). Altered aphid performances have been found on Bt-transgenic plants when compared with their non-transformed counterparts in some studies (Faria, Wäckers, Pritchard, Barrett, & Turlings, 2007; Lumbierres, Albajes, & Pons, 2004) but not in others (Lawo, Wäckers, & Romeis, 2009; Wolfenbarger, Naranjo, Lundgren, Bitzer, & Watrud, 2008). Studies with transgenic winter wheat expressing the coat protein gene from Barley yellow dwarf virus were reported to be superior hosts for aphids when compared with the non-transformed parental plant (Jimenez-Martinez & Bosque-Perez, 2009). These studies were all conducted either on the aphid population level or without taking into account aphid genotypes. The performance of aphids, however, does not only depend on the host plant but also on the aphid genotype, here referred to as clone.

The cyclical parthenogenetic life cycle of aphids gives rise to distinct clone lines. Several studies have shown that life-history parameters can vary among aphid clones (Dedryver, Hulle, Le Gallic, Caillaud, & Simon, 2001; Moran, 1991; Vorburger, 2005). Frequently, clones differ in their response to various environmental factors such as parasitoid attack (Ferrari, Muller, Kraaijeveld, & Godfray, 2001; Gwynn, Callaghan, Gorham, Walters, & Fellowes, 2005; Henter & Via, 1995; von Burg, Ferrari, Muller, & Vorburger, 2008) and pesticide application (Foster et al., 1997) but also host plant quality (Ferrari, Godfray, Faulconbridge, Prior, & Via, 2006; Mackenzie, 1996). This variation in response to environmental factors is referred to as genotype × environment interaction (G × E) (Falconer, 1952; Via, 1991) and implies that different environments, such as host plants, favour different clones.

In this study we hypothesized that bottom-up effects due to the genetic modification of the wheat lines can change the performance of individual aphid clones. To our knowledge, this is the first study to look at effects of transgenic disease-resistant wheat on the clonal performance of aphids. We compared a range of life-history parameters of 30 clones of the agriculturally important rose-grain aphid *Metopolophium dirhodum* Walker (Aphidinae: Macrosiphini) grown on four different transgenic wheat lines (*Triticum aestivum* L.) with a resistance against powdery mildew (*Blumeria graminis* f.sp. *tritici*) to their corresponding non-transgenic isolines and looked for genotype × environment interactions. Hereby, the 30 aphid clones were treated as the genotypes and the eight wheat lines as their environments.

Material and methods

Aphid clones

During summer 2007 we collected 30 clones of *M. dirhodum* from seven wheat fields around Zurich,

Switzerland. The minimum distance between any two of the collection sites of clones within the same field was at least 10 m, which was considered sufficient to avoid collecting the same clone more than once. From each sample, a single parthenogenetic female was selected to establish a clone line. The clone lines were kept on seedlings of the commercially available winter wheat variety Camedo. The caged plants with the aphid clones were kept in a climate chamber with a light:dark regime of 16:8 h, at a constant temperature of 22 °C with a relative humidity of 60%. These conditions ensured continuous parthenogenesis. Depending on their collection date, the aphid clones were in culture for approximately 6–12 parthenogenetic generations before the experiment began. More detailed information about the clone lines is provided in Appendix A.

Wheat lines

We used four spring wheat lines carrying the transgene *Pm3b* of wheat (*Pm3b* #1–4) which confers specific resistance to wheat powdery mildew (Srichumpa, Brunner, Keller, & Yahiaoui, 2005; Yahiaoui, Srichumpa, Dudler, & Keller, 2004) and their respective non-transgenic control lines (*S3b*#1–4). The four transgenic and their control lines are in the following referred to as wheat pairs #1–4.

The wheat lines had been generated by biolistic transformation of the wheat cultivar Bobwhite SH 98 26 (Pellegrineschi et al., 2002). The *Pm3b* gene was cloned from hexaploid wheat and expressed under the control of the maize ubiquitin promoter (Christensen & Quail, 1996). Transformants were selected on mannose containing media using the phosphomannose isomerase (PMI) coding gene as selectable marker (Reed et al., 2001). After regeneration of independent T0 transformants, four T1 segregants were selected for presence (transgenic lines *Pm3b* #1–4) or absence (control lines *S3b* #1–4) of the *Pm3b* transgene based on Southern hybridization (Southern, 2006). The transgenic lines contained one complete copy of the *Pm3b* (and an additional fragment in *Pm3b* #4) which segregated as a single Mendelian locus in the T1 generation. Homozygous lines of the transgenic and the control sister lines of the fourth generation of sexual reproduction (T4 seeds) were used in the experiment. Phenotypic expression of the resistance trait in the GM wheat lines used in our study was confirmed in a companion study by S. Zeller et al. (unpublished data).

Experimental set-up

We followed the fate of five individual aphids per clone on each wheat line (30 clone lines × 8 wheat lines × 5 replicates). The five replicates were assigned to five temporal blocks. Each block contained one replicate per clone × wheat line combination and was kept in the same climate chamber. Within block, pots were positioned randomly. Experimental plants were watered twice weekly; once a week a commercial fertiliser was added to the water (Wuxal; Maag AG, Dielsdorf, Switzerland; 100 g N, 100 g P_2O_2, 75 g K_2O/L). Owing to a significantly reduced germination success for wheat pair #4 ($F_{3,32} = 14.79$, $p < 0.000$), the actual number of individuals which comprised the experiment was 1081 instead of the 1200 that would have been required for a fully orthogonal design.

A single first instar aphid nymph was placed on each plant. The used nymphs were all born within 16 h. At the time of aphid addition, wheat plants had reached growth stage 12 according to Zadoks' scale (Lancashire et al., 1991; Witzenberger, Hack, & Van den Boom, 1989). At stage 12 the *Pm3b* transgene is already expressed (pers. comm., S. Brunner, Institute of Plant Biology, University of Zurich, Zurich, Switzerland). Every 12 h, the nymphs were checked for ecdysis. After adult ecdysis, the total development time and aphid morph (i.e. winged or wingless forms) were recorded. Immediately afterwards we weighed each aphid on a microbalance (Mettler Toledo, MT5, Greifensee, Switzerland) to the nearest 0.001 mg and returned it to its host plants. Each day, aphid mortality was checked and twice a week all offspring were counted and removed.

The life-history parameters measured and analysed were developmental time, adult weight, total offspring number and daily fecundity. Daily fecundity was deliberately defined as the number of offspring produced during the first seven days after having reached adulthood. Furthermore, an overall estimate of fitness was calculated following Service and Lenski (1982):

$$F_i' = \sum_{x=0}^{\infty} F_N{}^{-x} S_{xi} B_{xi}$$

F_N is an estimate of the finite rate of increase of the entire experimental population over the duration of one age class. Age class in this experiment refers to the periods in between the removal of the newborn aphid nymphs (i.e. 3.5 days in this experiment). F_N was iteratively obtained from Eq. (4) in Lenski and Service (1982). S_{xi} is the survivorship of the ith individual to age class x (either one or zero) and B_{xi} is the number of offspring born to the ith individual in age class x. Summarizing, F_i' is an estimate of the lifetime contribution of the ith individual to population growth. For further details about the calculation of F_i' see Service and Lenski (1982) and Vorburger (2005).

Data analysis

We analysed the five measured life-history parameters separately for the four different wheat pairs but also in

Table 1. Test statistics for the aphid clone and morph effect on the different life-history parameters. We used linear mixed models based on F statistics with aphid morph entering as a fixed effect and aphid clone as a random effect.

Source of variation		Nom. d.f.	Denom. d.f.	F	P
Clone	Development time	25	87	3.93	<0.001
	Adult weight	25	87	3.29	<0.001
	Daily fecundity	25	87	5.00	<0.001
	Total offspring number	25	87	5.41	<0.001
	F_i'	25	87	2.97	<0.001
Aphid morph	Development time	1	806	64.07	<0.001
	Adult weight	1	804	1.13	0.288
	Daily fecundity	1	776	51.68	<0.001
	Total offspring number	1	793	28.92	<0.001
	F_i'	1	806	70.81	<0.001

an overall analysis by using linear mixed models based on F statistics. In both analyses, the separate one and the overall one, a block effect (random) and aphid morph (fixed) were included as cofactors. The separate models for each wheat pair included two main effects which were GM (fixed) and clone (random) as well as their interaction GM × clone (random). The overall analysis included the main effects wheat pair (fixed), GM (fixed) and clone (random) as well as all their two- and three-way interactions.

Further, we calculated the correlation coefficients between mean clone performances on transgenic and control wheat lines for each of the four pairs as well as for overall GM and overall controls. Such correlations represent an approximation of the cross-environmental genetic correlation (Via, 1991; Via & Hawthorne, 2002).

At different steps in the experiment aphids died and therefore the sample sizes of the different analyses varied. None of the dependent variables were transformed, as they already met the assumptions of normality and homoscedasticity of residuals, except for total offspring number which was transformed to the power of two (y^2).

All the statistical calculations were done with the open source statistical software R version 2.7.0 for windows (R Development Core Team 2008).

Results

Of the 1081 aphids at the beginning of the experiment, only 31 aphids (less than 3%) did not survive to adulthood. These non-adult mortalities were independent of the wheat lines. As expected, we found significant variation among aphid clones for all life-history parameters and aphid morph influenced all the life-history parameters except adult weight (Table 1). Winged morphs did have a longer development time, a lower daily fecundity, produced less total offspring and had a lower estimate of overall fitness F_i'.

Pairwise comparison

With two exceptions we did not find any effect of the transgenic wheat lines on aphid life history when compared with the corresponding non-transgenic control lines for each of the four pairs of wheat lines (Fig. 1). The exceptions were development time which tended to be longer on the transgenic plants of wheat pair #1 compared with the control line (*Pm3b* #1 *vs. S3b* #1; $F_{1,213}=2.777$, $p=0.097$, Fig. 1a) and total offspring number which was significantly lower on transgenic than on control plants of wheat pair #4 (*Pm3b* #4 *vs. S3b* #4; $F_{1,139}=3{,}917$, $p=0.050$, Fig. 1d).

In no case did we find an aphid clone × GM interaction which means that the direction of the response variables was the same for the wheat lines within a pair. This was confirmed by the calculated correlation coefficients which did not reveal any trade-offs neither for any of the four wheat pairs nor for the overall GM effect compared with the control lines. Instead, most of the life-history parameters showed a significant positive correlation (Table 2).

Overall analysis

Similar results were found in the overall analysis (across all four wheat pairs). The overall factor GM significantly affected total offspring number ($F_{1,29}=8.70$, $p=0.006$). Aphids on the control plants produced 3.33% more offspring than aphids on the GM plants (total offspring number: 60 ± 0.71 vs. 58 ± 0.64). The remaining life-history parameters did not differ although there was a general tendency for an increased development time and a decreased adult weight on the GM plants compared with the control plants (development time: $F_{1,29}=3.60$, $p=0.068$; adult weight: $F_{1,29}=3.19$, $p=0.084$). None of the tested interactions was significant.

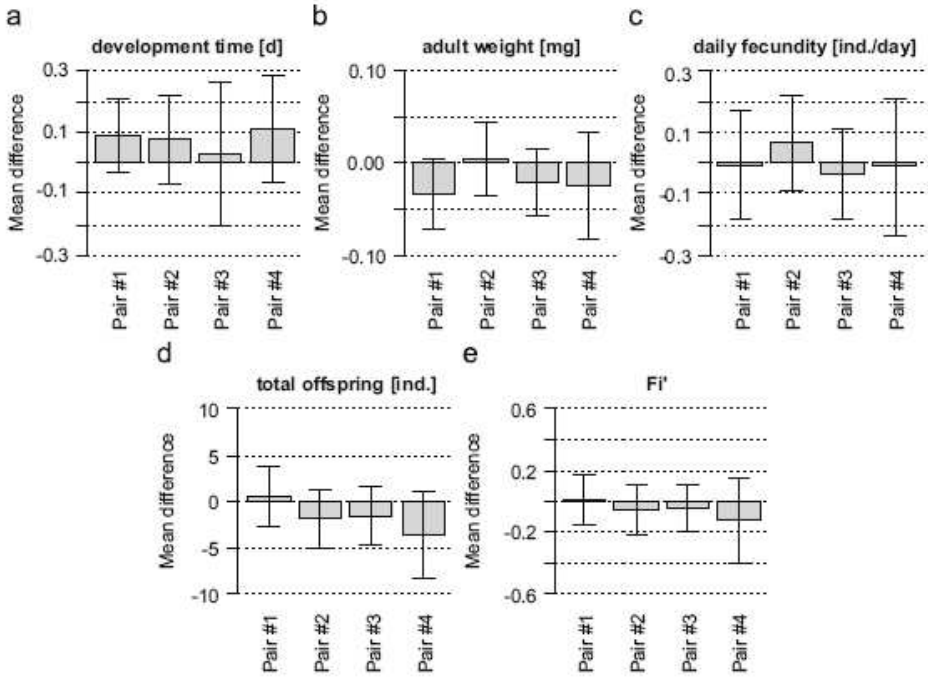

Fig. 1. Analysis of five *Metopolophium dirhodum* life-history parameters on four different *Pm3b*-transgenic wheat lines and their respective non-transgenic controls (wheat pair #1–4). Shown are the mean differences of the measures made on the transgenic plants and the control plants for (a) development time (days), (b) adult weight (mg), (c) daily fecundity (ind./day), (d) total offspring (ind.) and (e) F_i'. Error bars denote 95% confidence intervals. The mean differences were obtained by subtracting the values measured on the control plants from the values measured on the GM plants. Hence, negative differences stand for smaller measured values on the GM plants whereas positive differences are associated with higher measured values on the GM plants when compared with the control plants.

Table 2. Correlations of the clone means of *Metopolophium dirhodum* life-history parameters on four *Pm3b*-transgenic wheat lines and their corresponding non-transgenic control lines (wheat pairs #1–4) as well as for the data on overall GM versus overall controls. Shown are the correlation coefficients, *r*. All traits are positively correlated except F_i' for pair #4. Significant correlations are shown in bold and significance levels are indicated by asterisks.

Parameters	Pair #1	Pair #2	Pair #3	Pair #4	GM vs. control
Development time	**0.577*****	**0.545****	**0.355***	**0.383***	**0.818*****
Adult weight	0.037	**0.419***	0.181	0.377	**0.561*****
Daily fecundity	**0.455***	**0.397***	**0.468****	0.318	**0.725*****
Total offspring number	0.218	**0.612*****	**0.374***	0.282	**0.621*****
F_i'	**0.616*****	**0.431***	0.159	−0.040	**0.600*****

$^{*}p<0.05$.
$^{**}p<0.01$.
$^{***}p<0.001$.

Discussion

In this study we hypothesized that life-history variation among clones of the aphid *M. dirhodum* might be correlated with the occurrence of a transgene for mildew resistance in their wheat host plants. While we did find strong life-history variation among clones and weak evidence for transgene effects on total offspring number of aphids, there was no evidence for genotype × environment interactions for any of the life-history parameters measured on the aphids. Effects of the transgene on the performance of aphid clones could have been expected based on direct bottom-up effects such

as changed food quality of GM wheat. Indirect effects such as decreased mildew infection making GM plants more attractive to aphids could also result in an altered aphid performance (von Burg et al., unpublished data). However, in the present study we can rule out this possibility because the plants were kept under controlled conditions without mildew infection. Thus, the significantly lower number of offspring produced on the transgenic lines suggests that the transgene did lead to some metabolic changes that had bottom-up effects on the feeding aphids. However, the fact that we could not detect any variation among the 30 aphid clones further suggests that the nutrient quality of wheat for aphids was not perceivably altered by the genetic transformation.

The observation of significant genetic variation for all measured life-history parameters among the 30 aphid clones used in this study is consistent with other studies which found genetic variation among aphid clones for a range of traits (Dedryver et al., 2001; Ferrari et al., 2006, 2001; Gwynn et al., 2005; Henter & Via, 1995; Mackenzie, 1996; Moran, 1991; von Burg et al., 2008; Vorburger, 2005). Nevertheless, genetic variation in life-history traits and in particular in life-time fitness, is difficult to explain. In the long term or across a broader range of host environments the fitness ranking of the clones would have to change or the variation observed here should disappear due to selection. We point to the cited studies for discussion of the potential mechanisms that maintain such genetic variation in life-history traits.

To conclude we want to emphasize that studies of plant–insect interactions and their ecology are important to understand how these interactions shape insect herbivore communities. The use of transgenic plants opens up a whole range of new research questions, which are not necessarily questions about potential environmental risks of the novel plants (Raybould, 2010). In this study we wanted to find out if and how transgenic powdery mildew-resistant wheat affects the non-target aphid *M. dirhodum* and whether there are aphid clone × wheat lines (G × E) interactions. We could not detect any major effects of the transformed wheat line on a range of life-history parameters in this detailed laboratory study. This suggests that the genetic transformation did not alter the quality of the wheat plants as hosts for the aphid *M. dirhodum*.

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Appendix A. Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.baae.2009.02.003.

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Appendix A. Detailed list of the 30 *Metopolophium dirhodum* clone lines used in the experiment, including collection site, collection date and the average measures for the assessed life-history parameter ± SEM.

Clone	Collection site	Collection date	Development time [d]	Adult weight [mg]	Daily fecundity	Total offspring	F_i'
1.10	Katzensee	20.06.2007	8.15 ± 0.12	0.93 ± 0.02	4.41 ± 0.11	63.40 ± 2.05	2.67 ± 0.14
1.11	Reckenholz	28.06.2007	8.41 ± 0.15	0.96 ± 0.03	4.33 ± 0.16	64.00 ± 2.31	2.48 ± 0.19
1.12	Katzensee	20.06.2007	8.28 ± 0.13	0.93 ± 0.03	4.19 ± 0.15	49.26 ± 2.70	2.21 ± 0.15
1.13	Waidhof	23.06.2007	8.28 ± 0.13	0.97 ± 0.04	4.60 ± 0.14	64.81 ± 2.28	2.55 ± 0.15
1.14	Katzensee	24.06.2007	8.30 ± 0.14	0.97 ± 0.03	4.50 ± 0.16	59.73 ± 2.03	2.58 ± 0.14
1.15	Katzensee	24.06.2007	8.36 ± 0.13	0.99 ± 0.03	4.59 ± 0.13	61.70 ± 1.42	2.59 ± 0.13
1.16	Katzensee	24.06.2007	8.39 ± 0.13	1.00 ± 0.03	4.43 ± 0.17	51.40 ± 2.31	2.35 ± 0.18
1.17	Katzensee	24.06.2007	8.74 ± 0.13	0.97 ± 0.04	4.44 ± 0.16	59.38 ± 3.27	2.20 ± 0.16
1.18	Katzensee	24.06.2007	8.47 ± 0.14	0.90 ± 0.04	4.36 ± 0.17	57.82 ± 2.13	2.46 ± 0.15
1.19	Waidhof	23.06.2007	8.28 ± 0.13	1.02 ± 0.04	4.63 ± 0.16	63.19 ± 1.82	2.56 ± 0.18
1.20	Reckenholz	12.07.2007	8.78 ± 0.21	0.87 ± 0.03	3.87 ± 0.17	56.94 ± 3.46	1.95 ± 0.19
1.21	Reckenholz	12.07.2007	8.83 ± 0.38	0.96 ± 0.04	4.82 ± 0.13	58.76 ± 2.47	2.56 ± 0.14
1.22	Reckenholz	12.07.2007	8.39 ± 0.13	1.06 ± 0.03	4.63 ± 0.15	59.17 ± 1.95	2.66 ± 0.14
1.23	Reckenholz	12.07.2007	8.44 ± 0.19	0.83 ± 0.03	4.18 ± 0.18	61.63 ± 2.48	2.34 ± 0.14
1.26	Reckenholz	28.06.2007	8.27 ± 0.15	0.99 ± 0.04	4.02 ± 0.18	56.78 ± 3.28	2.25 ± 0.17
1.27	Reckenholz	05.07.2007	8.24 ± 0.17	0.88 ± 0.03	4.32 ± 0.14	51.44 ± 3.15	2.28 ± 0.18
1.28	Reckenholz	05.07.2007	8.21 ± 0.14	0.95 ± 0.03	4.51 ± 0.15	56.00 ± 2.17	2.49 ± 0.16
1.29	Reckenholz	05.07.2007	8.09 ± 0.15	0.96 ± 0.03	3.96 ± 0.18	61.91 ± 2.47	2.45 ± 0.16
1.30	Reckenholz	05.07.2007	8.85 ± 0.19	0.88 ± 0.02	3.69 ± 0.16	54.34 ± 2.44	1.78 ± 0.16
1.32	Reckenholz	05.07.2007	8.85 ± 0.17	0.96 ± 0.03	3.91 ± 0.18	56.03 ± 3.60	1.94 ± 0.17
1.33	Reckenholz	05.07.2007	8.00 ± 0.19	0.96 ± 0.04	4.36 ± 0.17	59.48 ± 2.89	2.64 ± 0.17
1.34	Reckenholz	05.07.2007	8.48 ± 0.20	0.92 ± 0.04	4.12 ± 0.17	56.44 ± 2.59	2.11 ± 0.15
1.35	Reckenholz	05.07.2007	8.50 ± 0.15	0.96 ± 0.03	4.37 ± 0.13	56.18 ± 2.04	2.22 ± 0.15
1.36	Reckenholz	05.07.2007	7.92 ± 0.16	1.02 ± 0.04	4.22 ± 0.14	63.05 ± 2.60	2.70 ± 0.17
1.37	Waidhof	23.06.2007	7.93 ± 0.14	1.02 ± 0.03	4.34 ± 0.18	60.26 ± 3.07	2.57 ± 0.14
1.38	Reckenholz	28.06.2007	8.47 ± 0.17	0.93 ± 0.04	4.16 ± 0.19	64.31 ± 2.80	2.22 ± 0.17
1.39	Käferberg	22.06.2007	8.37 ± 0.16	0.96 ± 0.02	4.16 ± 0.15	61.00 ± 1.99	2.41 ± 0.13
1.40	Reckenholz	28.06.2007	8.06 ± 0.13	0.97 ± 0.03	4.15 ± 0.16	64.69 ± 3.18	2.48 ± 0.16
1.41	Käferberg	22.06.2007	8.42 ± 0.14	0.89 ± 0.04	4.28 ± 0.18	50.94 ± 3.07	2.09 ± 0.18
1.42	Reckenholz	28.06.2007	8.02 ± 0.12	0.96 ± 0.03	4.08 ± 0.17	58.13 ± 3.28	2.06 ± 0.19

CHAPTER 3

Effect of transgenic powdery mildew-resistant wheat (*Triticum aestivum* L.) on naturally occurring insect herbivores in different environmental conditions

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Infestation of transgenic powdery mildew-resistant wheat (*Triticum aestivum* L.) by naturally occurring insect herbivores in different environmental conditions

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Abstract

Novel genetically modified (GM) plants with enhanced resistance to fungal diseases are being developed and the use of GM wheat plants with enhanced resistance to powdery mildew being explored as an alternative to the use of potent chemical fungicides. Growing GM crops always raises concerns about effects on non-target organisms. The aim of our study was therefore to assess the impact of transgenic disease-resistant wheat on the most common wheat herbivores, namely cereal aphids, the cereal leaf beetle, *Oulema melanopus*, and the chloropid gout fly, *Chlorops pumilionis* in the field and a convertible glasshouse. We used a range of different GM wheat lines as well as conventional wheat varieties and barley and triticale. There was significant annual variation and both aphid densities and powdery mildew infections were higher in the convertible glasshouse, whereas cereal leave beetle abundance and chloropid gout fly damage were more pronounced in the field. In the convertible glasshouse we found significantly more aphids on transgenic Pm3b*#1* plants compared to their control line. We assume that the reduced powdery mildew infection of these plants caused this effect and that the infection levels in the field were too low to detect such an effect there. Apart from this indirect effect which fall within the natural variation between wheat varieties and crop species we did not find differences between GM plants and their respective control plants.

1. Introduction

Genetically modified (GM) crops with enhanced resistance to insect pests and/or tolerance to broad-spectrum herbicides were first commercially released in 1996. Since then, the area devoted to such GM crops has continuously increased, reaching 134 million hectares in 2009 (James 2009). Currently, a range of novel GM plants with tolerance to different biotic stresses, altered composition, or those producing plant-derived pharmaceuticals are being developed (Sanvido et al. 2006; Stein & Rodríguez-Cerezo, 2010). In addition, a number of crops have been genetically engineered to enhance their resistance to fungal pathogens (Punja, 2001; Campbell et al. 2002).

Fungal diseases cause devastating losses in wheat (*Triticum aestivum* L.) worldwide (Wiese 1991) and, among these, powdery mildew, *Blumeria graminis* f.sp. *tritici*, is considered one of the most consistently damaging pathogens in Europe (Bliffeld et al. 1999). The use of GM wheat plants with enhanced resistance to powdery mildew is being explored as an alternative to the use of potent chemical fungicides (Bliffeld et al. 1999; Clausen et al. 2000). One of the concerns associated with the growing of GM crops is that that they could have adverse effects on non-target organisms (i.e., organisms that are not intended to be harmed by the trait under consideration) with potential implications for the sustainable deployment of the crop. Among these, herbivorous species are the ones most likely to be affected by GM plants, either directly or indirectly due to the expression of the trait or due to the transformation process.

The aim of our study was to assess the impact of several transgenic disease-resistant wheat plants on the performance of naturally occurring insect herbivores under field and semi-field environmental conditions. We have recorded the most common wheat herbivores, namely aphids (Hemiptera: Aphidae), the cereal leaf beetle, *Oulema melanopus* L. (Coleoptera: Chrysomelidae), and the chloropid gout fly, *Chlorops pumilionis* Bjerk. (Diptera: Chloropidae). GM wheat plants, either resistant against specific powdery mildew races or with a broad effect on all chitin containing fungi, were used in the experiments. Conventional

wheat varieties as well as barley (*Hordeum vulgare* L.) and triticale (x *Triticosecale* Wittmack) were used for comparison.

2. Material and Methods

2.1 Plant material

Six GM spring wheat lines carrying two different types of resistance genes that confer protection against powdery mildew were used. Four independent transformation events carrying the transgene *Pm3b* of wheat providing race-specific resistance to wheat powdery mildew (Pm3b*#1-4*) (Sricumpa et al. 2005; Yahiaoui et al. 2004), and their respective null-segregant lines (Sb*#1-4*) as non-transformed controls were used. These lines were generated by biolistic transformation of spring wheat cultivar Bobwhite SH 98 26 that has no endogenous *Pm3* gene and is, in general, sensitive to powdery mildew (Pellegrineschi et al. 2002). The Pm3b plants are further described in Brunner et al. (accepted). In addition, two transgenic Frisal lines expressing chitinase [Chi (A9)], and chitinase and glucanase [Chi/Glu (A13)] from barley, which should provide a broad active antifungal resistance (Zhu et al. 1994), and their non-transgenic control line, the Swiss spring variety Frisal, were deployed (Bliffeld et al. 1999). However, it has been shown that the resistance against powdery mildew is not increased in these two lines (Bieri et al. 2003). Additionally, the commercial wheat varieties Bobwhite, Casana, Fiorina, Rubli and Toronit, as well as the spring barley variety Estana and the spring triticale variety Trado were used for conventional comparison.

2.2 Convertible glasshouse studies

2.2.1 Experimental set-up

Experimental wheat plants were grown during the seasons 2008 and 2009 in a convertible glasshouse located at the Agroscope Reckenholz-Tänikon Research Station ART in Zurich (Switzerland). The research station is located just outside the city of Zurich in a rural area

surrounded by fields, forests and orchards. The glasshouse provides close to field conditions by exposing the plants to outside environmental temperatures and allowing natural colonization by insects and pathogens (Romeis et al. 2007). The experiment involved five entries: Pm3b*#1*, Sb*#1*, Chi/Glu (A13), Frisal and the spring wheat variety Rubli as a conventional check. Experimental wheat plants were grown in 40 plots (80 cm x 60 cm x 80 cm) arranged in two rows. Each wheat line was replicated eight times, assembling the replicates as blocks of five adjacent plots containing the five entries in randomized order. Each plot consisted of a separated central cylinder (26 cm diameter) for the experimental plants and a surrounding area containing buffer plants (i.e., non-transformed plants of the same variety) simulating a near-field situation. Twenty seeds were planted per central cylinder of each plot. After seedling emergence only ten experimental plants were left to grow. The wheat plants were grown for a period of about four months (sowing: 2.4.2008 and 19.3.2009; harvest: 30.7.2008 and 22.7.2009). Before sowing, basic fertilizer was added to the soil (per plot: 5.8g P. 7.2g K, 1.7g Mg, 8.7g N in 2008; 10.32g P, 8g K, 9g $Mg(NO_3)_2$ in 2009). Additionally, on May 5 and 28 in 2008, and on April 29 in 2009 each plot received 5-7 g of Ammonsalpeter (25% N, 5% Mg, 8.5% S). Plants were watered as needed. Pergamyn-paper bags (Franz Grätzer & Co., Einsiedeln, Switzerland) were placed over individual flowering ears of transgenic and control plants to prevent pollen from escaping the system. In the following, ‘plot’ will be referred to the central cylinder containing the 10 experimental plants.

2.2.2 Assessment of powdery mildew infection

Natural powdery mildew infection was scored once a week on the plot level for 6 consecutive weeks starting with the first occurrence of mildew. The Cobb’s scale ranging from 0 to 9 (0 = no symptoms, 9 = fully diseased) was used (Peterson et al. 1948). From this data the area under the disease progress curve (AUDPC) was calculated (Jeger and Viljanen-Rollinson

2001; Shaner and Finney, 1977). AUDPC was determined with data from 9 and 6 sampling dates in 2008 and 2009, respectively.

2.2.3 Monitoring of insect populations and damage

Abundance of aphids and larvae of the cereal leaf beetle *O. melanopus* was estimated by visual counts. Aphids were recorded separately for each species found. Samplings were conducted weekly between May 8 and July 17 in 2008, and between May 20 and July 9 in 2009. On each sampling date, all the experimental plants from all plots were inspected, and the data were subsequently pooled for each plot. After *O. melanopus* disappeared in early July, the typical feeding damage caused by the larvae was determined using a modified scheme developed for scoring infection with yellow rust (*Puccinia striiformis*) (Romeis et al. 2006). Leaf damage was scored using a scale from 0 to 6 based on the percentage of the leaf surface damaged (0 = no damage, 6 = over 75% of the leaf area damaged). For each plant, the flag and the second leaf from two tillers were monitored and the average damage scored was calculated per plot.

2.3 Field studies

2.3.1 Experimental set-up

The field surveys were performed during the 2008 and 2009 growing seasons at Agroscope ART. The field site was only about 300 meters apart from the convertible glasshouse and hence is located in the same rural area. In 2008, the experiment involved 18 different entries: Pm3b*#1-4*, their non-transgenic control lines Sb*#1-4*, the transgenic lines Chi (A9) and Chi/Glu (A13) and their control line Frisal, the conventional spring wheat varieties Bobwhite, Casana, Fiorina, Rubli and Toronit as well as the barley variety Estana and the triticale variety Trado. In 2009, 12 entries were used: Pm3b*#1-2*, Sb*#1-2*, Chi (A9) and Chi/Glu (A13), Frisal,

the conventional varieties Bobwhite, Rubli and Toronit, as well as barley (Estana) and triticale (Trado).

The plants were tested in a complete randomized block design with four replications, resulting in a total of 72 plots in 2008, and in five replications, resulting in a total of 60 plots in 2009. Plot size was 3.0 m × 1.3 m in both years. All seeds were treated with the fungicide Jockey (167 g l^{-1} Fluquinconazole, 34 g l^{-1} Prochloraz; Omya Agro AG, Safenwil, Switzerland) before sowing, and a total of 400 viable seeds per m^2 were sown in March. Spreader rows between the plots were sown to increase natural powdery mildew infection but no artificial inoculation was made. Fertilizer was administered at a rate of 46 kg P_2O_5 ha^{-1} and 60 kg K_2O ha^{-1} in autumn 2007 and 2008. Additionally, 30 kg N ha^{-1} was applied in 2008 shortly after sowing and at BBCH 39 (Witzenberger et al., 1989; Lancashire et al., 1991). In 2009, the same rate of nitrogen fertilizer was administered the day before sowing and at the phenological stage BBCH 22-29. All plots were sprayed with the herbicide cocktail Concert SX (40% Thifensulfurone, 4% Metusulfurone-methyl; Stähler Suisse AG, Zofingen, Switzerland) and Starane super (120 g l^{-1} Bromoxynil, 120 g l^{-1} Ioxynil, 100 g l^{-1} Fluroxypyr-metilheptil-ester; Omya Agro AG, Safenwil, Switzerland) in the beginning of May. In both years experimental plants were harvested beginning of August.

2.3.2 Assessment of powdery mildew infection

Natural powdery mildew infection was determined by estimating the percentage of the leaf surface infected. A total of 20 randomly chosen tillers per plot were inspected every second week, starting at the beginning of May. The data for each plot were subsequently pooled. The AUDPC was calculated with data from 5 sampling dates.

2.3.3 Monitoring of insect populations and damage

Abundance of aphids and *O. melanopus* larvae was estimated by visual counts. Samplings were conducted every second week between May 6 and July 29 in 2008, and between May 6 and July 14 in 2009. On each sampling date, insect abundance was assessed on the same 20 tillers per plot as mildew infection and the data were pooled for each plot. Additionally, the damage caused by the larvae of *O. melanopus* was determined after larvae had disappeared in early July, using the same score scheme as in the convertible glasshouse. For each plot, the flag leaf and the second leaf from 20 randomly chosen tillers were monitored and the average damage scored was calculated per plot. Finally, the damage produced by the larvae of the chloropid gout fly *C. pumilionis* was estimated. At the end of the growing season 30 randomly chosen tillers from different plants were checked per plot and the percentage of damaged plants was determined.

2.4 Data analysis

The data sets from the convertible glasshouse and the field were analysed separately. Natural powdery mildew infection, cumulative numbers of aphids and cumulative numbers and damage of *O. melanopus* larvae recorded in the convertible glasshouse were compared using two-way analyses of variance (ANOVA), with “plant line” and “year” as cofactors. For the parameters registered in the field, pair-wise comparisons between Pm3b plants and their respective controls were conducted. A two-way ANOVA was applied for the Pm3b/Sb*#1-2* pairs, whereas a one-way ANOVA was used to compare the Pm3b/Sb*#3-4* pairs, as those plants were only grown in 2008. Comparisons among the transgenic Chi (A9) and Chi/Glu (A13) and their control Frisal plants were conducted using a two-way ANOVA. Finally, field data sets from the different conventional wheat varieties and barley and triticale were compared using one-way ANOVA for 2008 and 2009 separately, as different wheat varieties were planted in both seasons. Whenever a two-way ANOVA was applied, “plant line” and “year” were used as cofactors. Except for the pair-wise comparisons, mean values were

subsequently separated using Tukey HSD-test. Correlation between aphid abundance and powdery mildew infection was calculated separately in the convertible glasshouse and in the field, whereas correlation between *O. melanopus* larval abundance and damage was analysed together for both environmental systems. Data of aphid abundance and natural powdery mildew infection in the field was square root transformed and damage by of *C. pumilionis* was arcsine transformed to meet model assumptions. Statistical analyses were conducted using the software package Statistica (Version 9.1, StatSoft Inc., Tulsa, OK, USA).

3. Results

3.1 Convertible glasshouse studies

3.1.1 Powdery mildew infection

Taking into account that scoring was conducted at 9 and 6 time points in 2008 and 2009, respectively, mildew infection was similar across both years of study (Fig. 1). Significant differences were observed among varieties, with Sb*#1* (non-transformed Bobwhite) being by far the most susceptible wheat line, followed by Frisal, and Rubli as the least susceptible line ($F_{4,65}$ = 101.96, $p < 0.001$) (Fig. 1). Transgenic Pm3b*#1* plants were significantly less infected than Sb*#1* plants, while powdery mildew infection levels did not differ between Chi/Glu (A13) and its control line Frisal (Fig. 1).

3.1.2 Monitoring of insect populations and damage

The cereal aphid species *Metopolophium dirhodum* (Walker), *Rhopalosiphum padi* (L.) and *Sitobion avenae* (F.) were found in the convertible glasshouse across both years, although significantly more aphids were recorded in 2009 (37.8 *vs.* 16.1 aphids/plant). In both years aphids started to infest the wheat plants by mid-May. Densities then increased to reach their maximum of 24.8 and 151.5 aphids/plant in early July 2008 or late June 2009, respectively, after which aphid numbers drastically declined. In both seasons, *M. dirhodum* was the most

common species (8.9 and 28.4 aphids/plant in 2008 and 2009, repectively), followed by *R. padi* (6.9 and 7.7 aphids/plant) and *S. avenae* (0.2 and 1.7 aphids/plant). Cumulative numbers of aphids/plant recorded on the different wheat lines are shown in Fig. 2a. Aphid abundance was similar among the three non-transgenic lines across both years. In contrast, significantly more aphids were recorded on transgenic Pm3b*#1* than on non-transformed Sb*#1* plants ($F_{4,65}$ = 3.00, p = 0.025). When the three aphid species were analyzed separately, only *M. dirhodum* abundance was significantly different between Pm3b*#1* and Sb*#1* ($F_{4,65}$ = 5.72, p < 0.001). Across all entries, aphid densities were negatively correlated with powdery mildew infection (p = 0.02; R = -0.35).

Very low densities of *O. melanopus* larvae were observed in the convertible glasshouse (an average of 0.17 and 0.21 larvae/plant in 2008 and 2009, respectively). In both seasons cereal leaf beetle larvae were first recorded in mid-May and observed for the following six weeks, disappearing at the end of June. Densities reached a maximum of 0.30 and 0.42 larvae/plant in early June 2008 and late May 2009, respectively. Cumulative numbers of *O. melanopus* larvae did not differ significantly among the wheat varieties and between transgenic Pm3b*#1* and Chi/Glu (A13) and their respective controls (Fig. 2b). Leaf damage caused by *O. melanopus* larvae was significantly higher on Rubli compared to Bobwhite and Frisal ($F_{4,65}$ = 8.27, p < 0.001). Nevertheless, average damage scores translate to leaf surface damage levels below 5% in both seasons, indicating that infestation by the beetles was very low.

3.2 Field studies

3.2.1 Powdery mildew infection

Low mildew infection levels were observed in the different wheat varieties in both seasons (Fig. 3). Bobwhite plants were much more infected compared to the rest of the conventional wheat lines as well as to barley and triticale in 2008 ($F_{7,24}$ = 5.71; p < 0.001), whereas no significant differences were observed in 2009. Decreased mildew infection levels were

recorded in transgenic Pm3b compared to their respective non-transformed Sb plants (Pm3b*#1* vs. Sb*#1*: $F_{1,14} = 22.7$; $p < 0.001$; Pm3b*#2* vs. Sb*#2*: $F_{1,14} = 6.18$; $p < 0.026$; Pm3b*#3* vs. Sb*#3*: $F_{1,6} = 11.44$; $p < 0.015$), except for the pair Pm3b*#4* vs. Sb*#4* ($F_{1,6} = 4.53$; $p < 0.077$), whereas similar infection levels were observed between Chi (A9), Chi/Glu (A13) and non-transformed Frisal plants.

3.2.2 Monitoring of insect populations and damage

In both growing seasons we found the same cereal aphid species as in the convertible glasshouse: *M. dirhodum*, *R. padi* and *S. avenae*. First aphids were recorded in early May and densities subsequently increased to reach a maximum towards the end of June (1.19 and 2.38 aphids/tiller in 2008 and 2009, respectively). After that, aphid populations rapidly declined and, by mid-July, hardly any aphids were observed on the plants. Throughout the course of the season, higher aphid densities were registered in 2009 compared to 2008 (0.67 vs. 0.45 aphids per tiller, respectively) (Fig. 4A). *Metopolophium dirhodum* (0.27 and 0.30 aphids/tiller in 2008 and 2009) and *S. avenae* (0.15 and 0.37 aphids/tiller) were the most dominant species, whereas *R. padi* was very scarce (0.03 and 0.01 aphids/tiller). Neither were there significant differences between the wheat varieties and barley and triticale nor between the transgenic plants and their respective controls (Fig. 4a). Overall, aphid abundance across all wheat lines was not found to correlate with powdery mildew infection.

Oulema melanopus larvae were much more abundant in 2008 compared to 2009 (0.48 and 0.09 larvae/tiller) (Fig. 4B). In both growing seasons, highest densities were observed in early June (0.98 and 0.19 larvae/tiller in 2008 and 2009, respectively) and larvae had completely disappeared by early July. Damage was also higher in 2008 than in the subsequent year, with average scores of 1.7 and 1.0, respectively. Leaf surface damage was thus below 15% and 5% in the two years. Statistical analysis revealed that neither densities of *O. melanopus* nor the damage caused by the larvae differed significantly among the conventional wheat varieties

and barley and triticale plants, and between the transgenic lines and their respective controls (Fig 4b). *Oulema melanopus* larval abundance and damage recorded in the convertible glasshouse and in the field were positively correlated ($p < 0.001$; $R = 0.87$) (Fig. 5).

The damage caused by *C. pumilionis* larvae in the field was much higher in 2008 compared to 2009, with 43% and 16% of the plants being damaged, respectively (Fig. 4C). No differences were detected among the different wheat varieties across both seasons, whereas fewer barley plants were damaged by *C. pumilionis* both in 2008 ($F_{7,24} = 6.57$, $p < 0.001$) and 2009 ($F_{5,24} = 11.95$, $p < 0.001$) compared to wheat and triticale plants (Fig. 4c). The damage caused by the chloropid gout fly larvae was similar between the transgenic lines and their respective controls (Fig. 4c).

4. Discussion

The main goal of this study was to investigate the effect of transgenic powdery mildew-resistant wheat plants on herbivorous insects under field and semi-field conditions. During two growing seasons, naturally occurring herbivores were recorded on several GM wheat plants and their respective non-transformed controls, as well as on conventional wheat varieties and barley and triticale plants.

Natural powdery mildew infection levels were much higher in the convertible glasshouse than in the field across both study years, probably due to differences in temperature and humidity between both environments. Similarly, Zeller et al. (2010) found increased mildew infection levels in the Sb lines grown in a controlled glasshouse compared to the field. Powdery mildew resistance was significantly increased in all Pm3b lines compared to their respective non-transformed controls, both in the convertible glasshouse and the field, as also shown by Zeller et al. (2010) and Brunner et al. (accepted). In contrast, transgenic Chi (A9) and Chi/Glu (A13) did not confer protection against the fungal pathogen and similar infection levels were

reported when compared to non-transformed Frisal plants, as already stated by Bieri et al. (2003).

In the convertible glasshouse, aphid abundance was negatively correlated with powdery mildew and resistant Pm3b*#1* plants harbored larger aphid populations compared to their susceptible controls. When the three aphid species were analyzed separately, differences were only observed for the most abundant species, *M. dirhodum*, whereas the two other aphid species recorded, *R. padi* and *S. avenae*, remained unaffected. In contrast, aphid densities did not differ between transgenic Chi/Glu (A13) plants and their Frisal control plants possibly because resistance against powdery mildew was not increased in the transgenic line. A previous laboratory study demonstrated that the *Pm3b* transgene has no major effect on the performance of individual *M. dirhodum* clones in the absence of powdery mildew (von Burg et al. 2010), suggesting that the higher aphid densities recorded on the transgenic Pm3b*#1* plants in our study can possibly be explained by the decreased mildew infection levels. However, the mechanisms underlying the negative effects of powdery mildew on aphids remain unclear and further evidence needs to be provided.

Interactions between insects and fungal pathogens sharing the same host are well documented and can take place directly between the two counterparts or can be mediated by the host plant (Hatcher 1995). The effects observed on *M. dirhodum* in our study might be due to the mycelium of the fungal pathogen covering the surface of infected leaves and, therefore, preventing the aphids from penetrating the plant tissue to reach the phloem sap. However, we cannot rule out that mildew indirectly affect *M. dirhodum* by inducing physiological changes in the susceptible wheat plants. It is known that fungal infection is associated with a reduced nutrient concentration within host plant tissue (Hatcher 1995), and could also affect phloem composition. Some studies have indicated that the nutritional quality of the phloem sap correlates positively with the performance and behavior of several aphid species (Karley et al. 2002, Ponder et al. 2002, Pescod et al. 2007). Hence, future experiments could be directed to

elucidate whether phloem sap composition differs between transgenic and control plants in absence and presence of powdery mildew.

Aphid densities registered in the field were much lower compared to the convertible glasshouse and were not correlated with mildew infection. Differences among the conventional lines and between any of the transgenic wheat plants and their respective controls were not reported. These results are, however, not surprising since, contrary to the convertible glasshouse, both aphid abundance and mildew infection levels were very low in both growing seasons.

Abundance and damage of *O. melanopus* larvae in the convertible glasshouse did not differ between either of the two transgenic lines and their respective non-transformed controls. Other studies have shown that chrysomelid beetles can be indirectly affected by fungal pathogens. Performance of larvae and adults of the green dock beetle *Gastrophysa viridula* (De Geer) feeding on rust-infected leaves of *Rumex crispus* L. and *Rumex obtusifolius* L. was reduced when compared to healthy plants (Hatcher et al. 1994). In this study, the observed effects were related to a lower nutritive quality of the infected leaves. Similarly, developmental time and weight of immature stages of the thistle tortoise beetle *Cassida rubiginosa* Müller was negatively affected when feeding on leaves of *Cirsium arvense* (L.) infected with a necrotrophic fungus compared to when feeding on control leaves (Kruess 2002). Reasons for the absence of powdery mildew-mediated effects in our study include, first, the fact that *O. melanopus* larvae appeared early in the season and completed their life cycle before susceptible plants were highly infected with powdery mildew, and second, the fact that larvae preferably feed on higher wheat leaves, which are much less infected than lower leaves.

Differences between transgenic plants and their respective controls were not reported in the field for the herbivores *O. melanopus* and *C. pumilionis* Lower damage levels of *C. pumilionis* were found in barley plants compared to wheat and triticale. A marked preference

of the chloropid gout fly for wheat in respect to barley has also been documented by Lilly (1947). Abundance and damage of *O. melanopus* were positively correlated, suggesting that the damage caused by larvae is a good representative for their abundance and could be sufficient to evaluate the effects of transgenic crops on *O. melanopus*, since this is less time consuming than conducting periodical visual samplings during the life cycle of the leaf beetle. Our study was conducted simultaneously across two growing seasons in a convertible glasshouse and a field, both located in the same rural area, which allows us to compare both environmental systems. While wheat plants grown in the convertible glasshouse were severely infected by powdery mildew and hosted high aphid densities, plants cultivated in the field showed minor symptoms of the fungus and aphids were very scarce. Probably for these reasons, the reported negative effects of powdery mildew-infected plants on aphid densities could not be detected in the field. Conditions in the convertible glasshouse seem to enhance mildew infection and aphid populations which could be an advantage for detecting differences in abundance. Nevertheless, the convertible glasshouse is an artificial system compared to the field and might suffer from limitations as the lack of cereal leaf beetles and the chloropid gout fly showed. Therefore field experiments still remain a necessity. However, considering that field trials with transgenic crops suffer from high additional costs due to government regulatory constraints and public opposition (Bernauer et al. 2011) the convertible glasshouse like the one used in our study seems to be a suitable environmental system to assess the impact of fungal resistant-transgenic plants on naturally occurring herbivores.

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FIGURE 1.

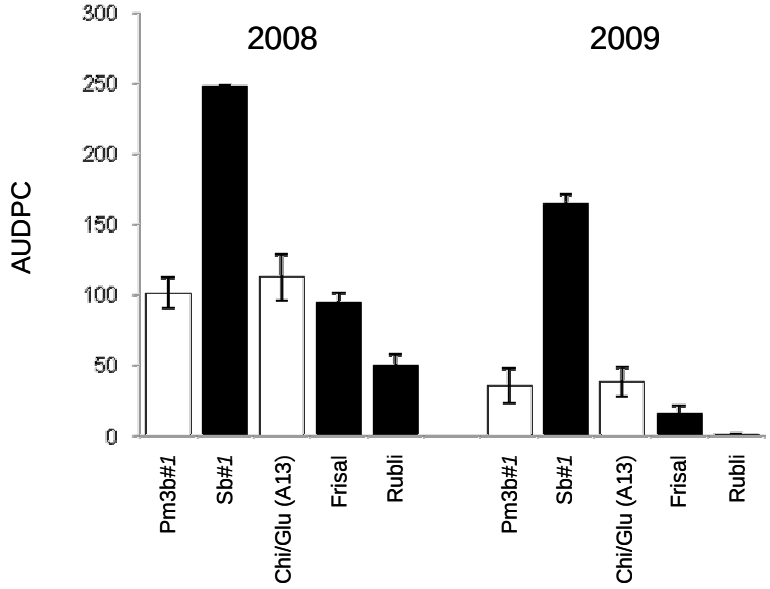

Fig. 1. Area under disease progress curve (AUDPC) (±SE) for powdery mildew infection of transgenic Pm3b#*1* and Chi/Glu (A13) lines, their respective non-transformed control lines Sb#*1* and Frisal, and the Swiss spring wheat variety Rubli grown in the convertible glasshouse in 2008 and 2009 (N = 8). Infection was scored using a 1 to 9 scale (1 = no symptoms, 9 = fully diseased). AUDPC was calculated with data from 9 and 6 sampling dates in 2008 and 2009, respectively. Therefore, AUDPC values are not comparable between years. White bars represent transgenic lines while black bars represent non-transformed lines.

FIGURE 2.

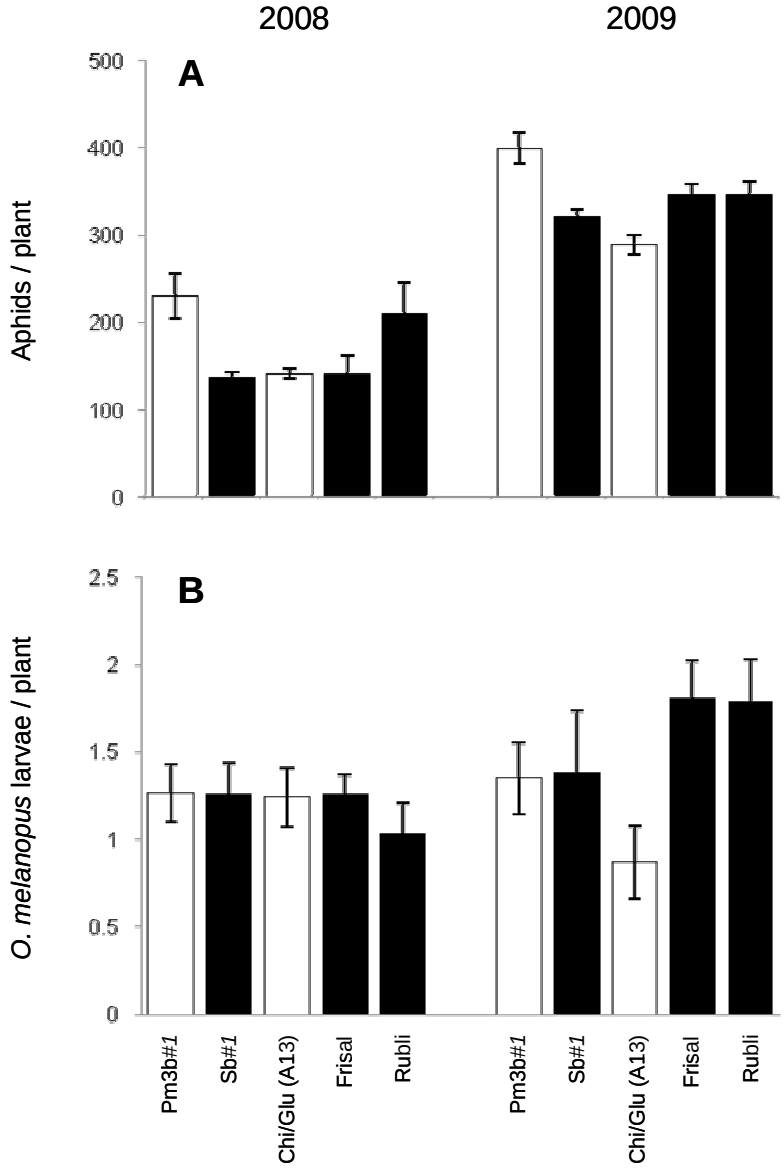

Fig. 2. Cumulative numbers (±SE) of (A) all aphid species and (B) *Oulema melanopus* larvae recorded in transgenic Pm3b#*1* and Chi/Glu (A13), their respective non-transformed control lines Sb#*1* and Frisal, and the Swiss spring wheat variety Rubli grown in the convertible glasshouse in 2008 and 2009 (N = 8). Cumulative numbers were calculated with 11 and 9 sampling dates in 2008 and 2009, respectively. White bars represent transgenic lines while black bars represent non-transformed lines.

FIGURE 3.

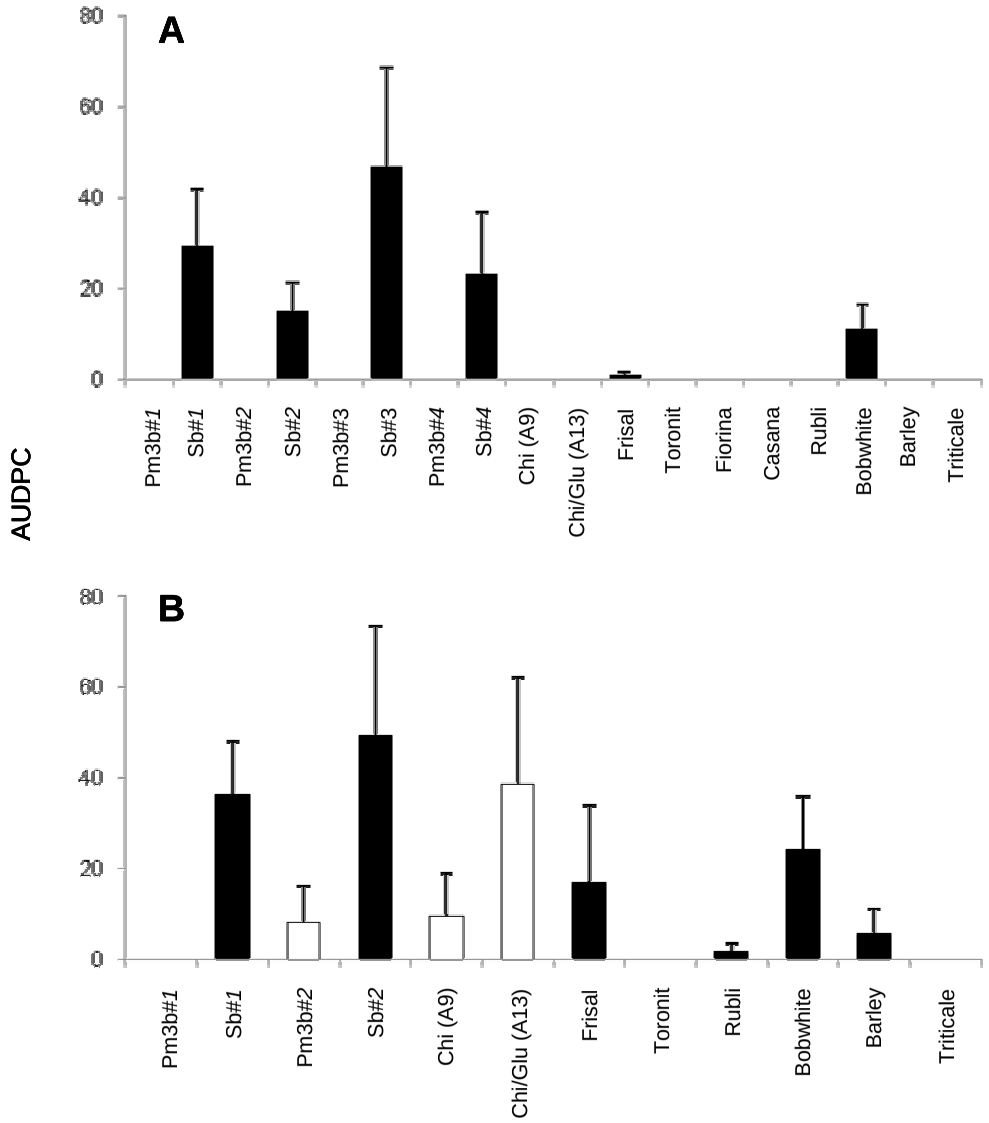

Fig. 3. Area under disease progress curve (AUDPC) (±SE) for powdery mildew infection of the different entries grown in the field in (A) 2008 and (B) 2009. Percentage of the leaf surface infected with powdery mildew was estimated. The AUDPC was calculated with data from 5 sampling dates in both years. White bars represent transgenic lines while black bars represent non-transformed lines.

FIGURE 4.

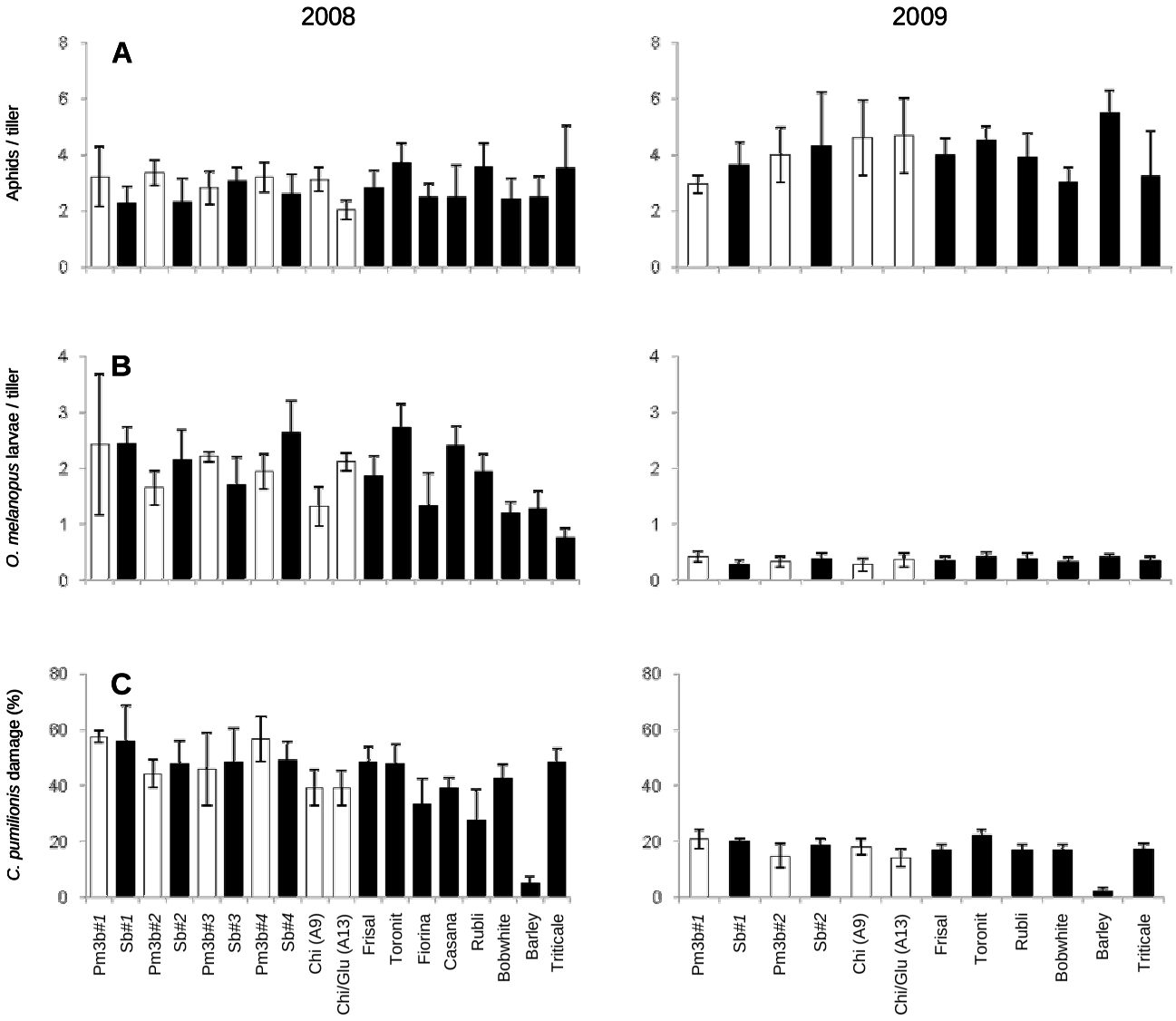

Fig. 4. Cumulative numbers (±SE) of (A) all aphid species, (B) *Oulema melanopus* larvae and (C) percentage (±SE) of damaged plants caused by *Chlorops pumilionis* in plants recorded in the different entries grown in the field in 2008 (N = 4) and 2009 (N = 5). Cumulative numbers were calculated with data from 7 and 6 sampling dates in 2008 and 2009, respectively. White bars represent transgenic lines while black bars represent non-transformed lines.

FIGURE 5.

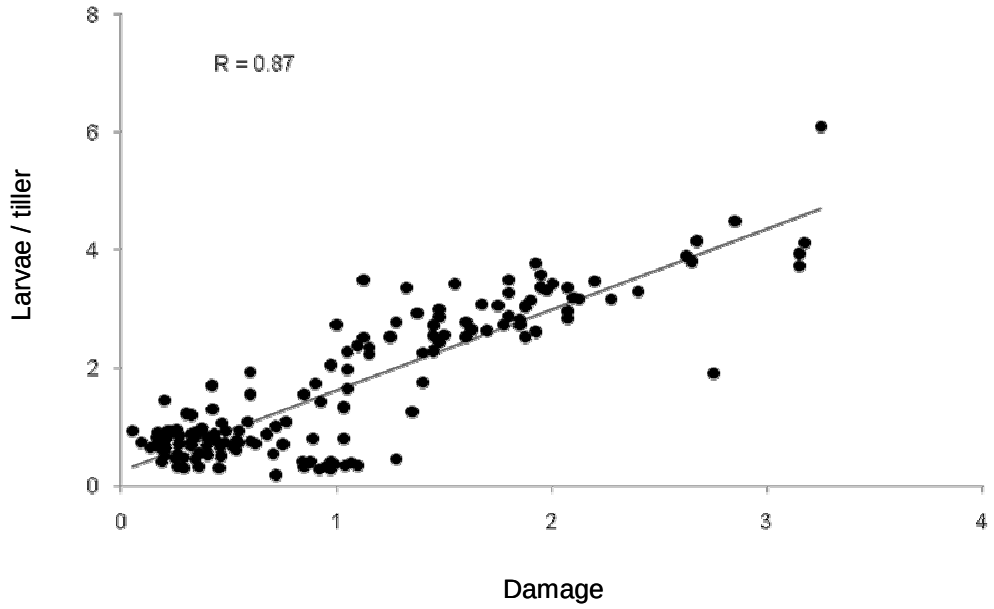

Fig. 5. Correlation between abundance (cumulative numbers per tiller) and damage of *Oulema melanopus* larvae recorded in the convertible glasshouse and in the field in 2008 and 2009.

CHAPTER 4

Protection from powdery mildew infection makes wheat a better host plant for some aphids

SIMONE VON BURG, FERNANDO ALVAREZ-ALFAGEME, & JÖRG ROMEIS

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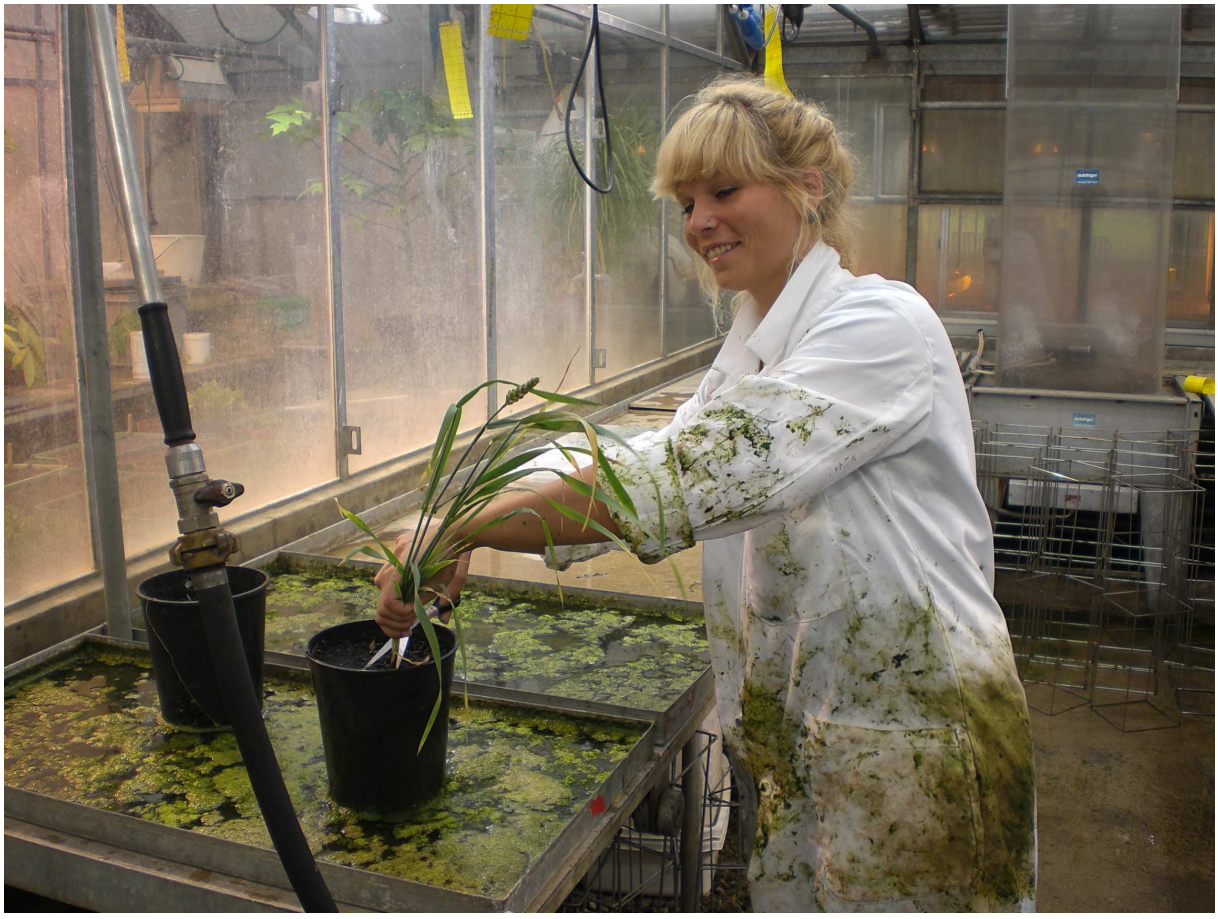

Protection from powdery mildew infection makes transgenic wheat a better host plant for some aphids

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Abstract

In agricultural ecosystems arthropod herbivores and plant fungal pathogens are widespread. The chances that herbivores and fungal pathogens encounter each other on the same plant are high and there they may alter conditions for each other. During the past decade numerous crop plants have been genetically engineered to enhance resistance to fungal pathogens. This provides an ideal opportunity for research of interactions between insect herbivores, fungal phytopathogens and their host plants. In this study we investigated the effects of powdery mildew of wheat on two cereal aphid species, *Metopolophium dirhodum* (Walker) and *Rhopalosiphum padi* (Linnaeus). We conducted three population experiments to distinguish between variety effects and effects caused by powdery mildew hypothesizing that aphids, feeding on infected plants grow slower and remain smaller compared to aphids on non-infected plants. We measured population size as well as individual size on infected and non-infected plants of different wheat varieties amongst which were two genetically modified (GM) powdery mildew-resistant lines. We found that only *M. dirhodum* was affected by powdery mildew resulting in reduced population numbers. Aphid populations on the transgenic mildew-resistant wheat plants were up to twice as big in some cases compared to populations on the non-transgenic controls which could be explained by decreased mildew levels. This is the first study to show indirect non-target effects of GM plants with a resistance against a fungal pathogen and implies that the control of one pest results in healthier plants which in turn could become more favourable for another pest and thus potentially limit their sustainable deployment.

Keywords: bottom-up effects, crop protection, genetically modified, pest management

Introduction

In agro-ecosystems, both insect herbivores as well as pathogenic fungi are abundant and the probability that they eventually colonise the same plant is high. Both depend on plant tissue and each of them may alter the conditions for the other party. Interactions can be direct, plant-mediated or both. Direct interactions include for instance feeding relationships. Occasionally, herbivores feed upon fungal pathogens or fungus-infected plant tissue (1, 2). On the other hand, damaged plant tissue caused by feeding herbivores, facilitate the entrance of fungal pathogens (3). Furthermore, insects act as dispersing vectors for fungi (4). Indirect plant-mediated interactions take place through modifications in the allocation of plant metabolites or trough plant defence mechanisms and can be caused by the herbivore or the pathogen (2, 5, 6). Either party can thus alter suitability and quality of the host plant for the other and often does this in a negative way (1).

Since the commercialization of genetically modified (GM) crops in 1996 one of the main concerns has been the effect on organisms that are not targeted by the GM trait. Insect-resistance is one of the dominant traits in GM crops and numerous studies have assessed the impact of insect-resistant crops expressing Cry proteins derived from the soil bacterium *Bacillus thuringiensis* (Bt) on insect herbivores (7) or natural enemies (8, 9). During the past decade, however, numerous crop plants have been genetically engineered to enhance resistance to fungal pathogens (10, 11). Even though, disease-resistant plants do not have insects as targets, the question about their effects on them remains since this could affect the sustainable production of the crop. However, compared to GM plants that express insecticidal proteins the mechanisms of such potential effects might change. Rather than being directly affected by a plant-produced toxin it is more likely that indirect effects through plant-pathogen interactions occur. In this study we investigated the effects of powdery mildew-resistant wheat on two non-target cereal aphid species, *Metopolophium dirhodum* Walker (Aphidinae: Macrosiphini) and *Rhopalosiphum padi* Linnaeus (Aphidinae: Aphidini).

Aphids belong to the world's significant agricultural pests (12, 13) and they react sensitively to metabolic and physiological changes in their host plants. Their performance can depend on phloem-sap composition (14-17) as well as on secondary plant metabolites (18-21). *Metopolophium dirhodum* and *R. padi* are globally distributed generalists. Both are cyclical parthenogens which alternate between their primary and their secondary host plants, amongst which we find major cereals such as wheat *Triticum aestivum* Linnaeus. As strict herbivores, aphids depend on plant tissue and phloem-sap throughout their whole lifes. Powdery mildew of wheat [*Blumeria graminis* (DC.) Speer var. *tritici*] is an obligate, biotrophic fungal pathogen and is widely distributed throughout the world. It especially thrives in cool, humid regions and if infection levels are high it ultimately leads to yield losses (12, 22). Powdery mildew is easy to spot as the mycelium forms a white, fluffy layer on the leaves. As an obligate, biotrophic pathogen, powdery mildew depends on the same host plant as aphids. Observations from a previous study that we conducted indicated that plants resistant to powdery mildew had higher aphid populations instead (23). We therefore hypothesized that aphids, feeding on plants infected with powdery mildew will grow slower and remain smaller compared to aphids on healthy plants. We performed population experiments, measuring aphid population size and aphid individual size on powdery mildew-infected and not infected plants of different wheat varieties amongst which were two GM wheat lines (Pm3b*#1* and Chi/Glu(A13)) and their respective controls (Sb*#1* and Frisal). The line Pm3b*#1* of the variety Bobwhite carried the *Pm3b* gene which confers specific resistance to powdery mildew (24, 25). The line Chi/Glu(A13) of the variety Frisal expressed chitinase/glucanase genes from barley which should confer a broad resistance against fungal pathogens (26). We used two different powdery mildew strains (strain A & strain V) of wheat from which one (i.e., strain V) is known to be able to break the resistance of the Pm3b*#1* plants (25) unlike strain A which cannot break the resistance. In the following we refer to plants that were treated with powdery mildew of either strain or that were not treated as "inoculated" and "not inoculated".

Plants that show powdery mildew symptoms as a consequence of the treatment are referred to as “infected”, those showing no symptoms as “not infected”.

By combining three different, consecutive experiments we were able to distinguish between wheat variety effects, effects of powdery mildew infection and to exclude unintended effects caused by the genetic modification on aphids. In the first experiment, the goal was, to compare effects of six commercially available wheat varieties on aphid performance in presence and absence of powdery mildew. The goal of the second experiment was to compare the GM wheat lines, Pm3b*#1* and Chi/Glu(A13) with their respective controls, Sb*#1* and Frisal, also in the presence and absence of powdery mildew. In these two experiments we used mildew strain A to inoculate the plants. In the third experiment we separated the GM effect from the effect caused by the powdery mildew infection by only using the two wheat lines Pm3b*#1* and Sb*#1* but including both mildew strains A and V. We will refer to the three experiments as the “Variety comparison”, the “GM/control comparison” and the “Bobwhite GM/control comparison”.

Results

MILDEW INFECTION AND C:N RATIO

The inoculated plants in the Variety comparison showed strong mildew infection symptoms, whereas the not inoculated plants remained healthy (Fig. 1a). In the GM/control comparison all the inoculated plants were infected with powdery mildew except for the transgenic Pm3b*#1* line. The introduced resistance worked for this GM line, whereas for the transgenic Chi/Glu(A13) plants it did not. This line was equally susceptible to powdery mildew as its non-transformed control line Frisal (Fig. 1b). The three treatments (not inoculated, inoculated with strain A and inoculated with strain V) in the Bobwhite GM/control comparison resulted in the expected infections of the wheat lines (Fig. 1c). The non-transformed control line Sb*#1* showed equally high infection levels for both mildew strains, whereas the not inoculated

plants remained healthy. As seen in the GM/control comparison the transgenic Pm3b*#1* was resistant to strain A. The plants of this treatment as well as the not inoculated plants did not show powdery mildew symptoms. However, strain V broke the resistance mechanism and was able to infect the transgenic Pm3b*#1* plants. There were some mildew cross-infections in both GM/control comparisons (Fig. 1b & 1c), yet infection levels were so low that we did not exclude these plants from the analysis.

To get a rough estimate about changes in plant metabolites we determined the C:N ratio of the different wheat lines in the three experiments. The C:N ratios ranged from 7.35 (± 0.31) to 9.59 (± 0.85) but did not reveal any treatment effects. Some variety effects were observed but only in the GM/control comparison ($F_{1,39}$ = 12.95, p < 0.001) with Frisal having a higher C:N ratio compared to Bobwhite (8.76 ± 0.23 vs. 7.77 ± 0.21).

APHID POPULATION SIZE

Variety comparison

Population size of *M. dirhodum* was significantly smaller on the powdery mildew-infected plants ($F_{1,74}$ = 12.10, p < 0.001) whereas *R. padi* remained unaffected by the treatment (Fig. 2a & 2b) and was rather influenced by the wheat variety ($F_{5,105}$ = 14.43, p < 0.001) and the biomass of the plant ($F_{1,105}$ = 47.04, p < 0.001) none of which influenced *M. dirhodum*.

GM/control comparison

To analyse the data of this experiment we built seven orthogonal a priori contrasts (C1-C7) which are described in Fig. 3a. The population size of *M. dirhodum* was significantly smaller on the inoculated, infected Frisal plants compared to the not inoculated, not infected Frisal plants ($F_{1,23}$ = 7.89, p = 0.010) (Fig. 3b, C3). Unexpectedly, we did not find significant differences between the inoculated but not infected Pm3b*#1* plants and the inoculated and infected Sb*#1* plants (Fig. 3b, C5). All the other contrasts were not significant as expected.

Again we found a significant variety effect on *R. padi* populations ($F_{1,55}$ = 14.32, p < 0.001). As in the Variety comparison, Bobwhite hosted much bigger aphid populations than Frisal (Fig. 3c, C1). We also found significantly more aphids on not inoculated Bobwhite plants compared to inoculated Bobwhite plants ($F_{1,55}$ = 7.61, p = 0.008) (Fig. 3c, C2). This probably resulted from a significantly and unexplainably bigger population size on the not inoculated Sb*#1* plants compared to the not inoculated Pm3b*#1* plants ($F_{1,55}$ = 5.44, p = 0.023) (Fig. 3c, C4). Plant biomass had a significant positive effect on both aphid species (*M. dirhodum*: $F_{1,23}$ = 13.45, p = 0.001; *R. padi*: $F_{1,55}$ = 42.84, p < 0.001).

Bobwhite GM/control comparison

The data of the Bobwhite GM/control comparison was also analysed with different orthogonal a priori contrasts which are described in Fig. 4a.

We found a significant treatment effect for M. dirhodum population size with the inoculated treatment having the biggest aphid populations, the plants inoculated with strain A intermediate aphid populations, and the plants inoculated with strain V the smallest populations ($F_{2,45}$ = 8.48, p < 0.001) (Fig. 4b, C1). Within the respective treatments, there was neither a difference between the not infected Pm3b*#1* and the not infected Sb*#1* lines in the not inoculated treatment (Fig. 4b, C2), nor between the both infected Pm3b*#1* and Sb*#1* lines inoculated with mildew strain V (Fig. 4b, C4). The only difference we found was within the treatment where we inoculated plants with mildew strain A resulting in not infected Pm3b*#1* plants and infected Sb*#1* plants ($F_{1,45}$ = 8.36, p = 0.006) (Fig. 4b, C3). Here the resistant Pm3b*#1* plants had bigger aphid populations opposed to not resistant Sb*#1* plants. These results confirm that powdery mildew infection is the crucial factor determining *M. dirhodum* population size and that there are no direct transgene effects.

Rhopalosiphum padi populations were less consistent. There was also an overall treatment effect ($F_{2,52}$ = 4.06, p = 0.023) but populations were biggest on the plants inoculated with

strain A (Fig. 4c, C1). No other differences were found. Again, plant biomass had a significant positive effect on both aphid species (*M. dirhodum*: $F_{1,48}$ = 33.10, p < 0.001; *R. padi*: $F_{1,52}$ = 95.66, p < 0.001).

APHID SIZE

Variety comparison

Both aphid species were smaller on the infected plants even though *R. padi* only marginally so (*M. dirhodum*: $F_{1,72}$ = 43.22, p < 0.001; *R. padi*: $F_{1,83}$ = 3.61, p = 0.061). The size of *M. dirhodum* was marginally influenced by wheat variety ($F_{1,72}$ = 2.23, p = 0.060) and positively associated with population size ($F_{1,72}$ = 29.88, p < 0.001). The aphids from large populations were also bigger in size. For *R. padi* the treatment × wheat variety interaction was significant ($F_{1,83}$ = 2.32, p = 0.050).

GM/control & Bobwhite GM/control comparisons

Individual aphid size of *M. dirhodum* was unaffected by treatment and wheat line in both experiments. Rather aphid size was positively correlated with population size (GM/control comparison: $F_{1,23}$ = 7.39, p = 0.012; Bobwhite GM/control comparison: $F_{1,48}$ = 11.69, p < 0.001). *Rhopalosiphum padi* size was completely unaffected in the GM/control comparison. In the Bobwhite GM/control comparison we found bigger aphids on the not inoculated non-transgenic Sb*#1* plants compared to the not inoculated GM Pm3b*#1* plants ($F_{1,52}$ = 4.67, p = 0.035). Further, in this comparison individual size of *R. padi* was also aphid density-related but in a negative way ($F_{1,52}$ = 3.91, p = 0.053).

Discussion

In this study we investigated how two aphid species were affected by the fungal pathogen powdery mildew of wheat. For this we used conventional wheat varieties as well as GM

wheat lines and we measured aphid population size and aphid individual size. It turned out that population size was the more sensitive and reliable parameter whereas individual size remained mostly unaffected by the treatment, showed inconsistent effects, or was aphid density-dependent.

The two aphid species reacted quite differently to the presence of powdery mildew and the wheat varieties. For *M. dirhodum* we confirmed our initial hypotheses regarding population size which was consistently smaller on powdery mildew-infected plants irrespective of whether they were commercial varieties or GM plants. This was true for two out of three experiments (Variety comparison & Bobwhite GM/control comparison) and partially so in the third one (GM/control comparison). In the GM/control comparison we did not find a significant difference between the resistant Pm3b*#1* plants and the Sb*#1* plants in the inoculation treatment (Fig. 3b, C5). Still, according to our expectations, there were more aphids on the Pm3b*#1* plants, yet this difference was not significant. We lost half of the replicates of *M. dirhodum* in this experiment due to an infection with an entomopathogenic fungus and thus the reduced statistical power may be responsible for the fact that we did not detect a significant effect as in the other experiments.

Rhopalosiphum padi population sizes were much less consistent and mostly unaffected by the powdery mildew treatment. Rather it was wheat variety and plant biomass which influenced the population growth of this aphid species. However, there were two effects that we are unable to explain. First, the almost doubled population size of *R. padi* on the not inoculated Sb*#1* plants compared to the not inoculated Pm3b*#1* plants in the GM/control comparison (Fig. 3c, C4). Since all the seven remaining experimental populations were of similar size (Fig. 3c) we think this is an artefact of some sort. We can also not explain the bigger population number on plants inoculated with powdery mildew strain A in the Bobwhite GM/control comparison (Fig. 4c, C1). It is neither possible to relate these effects to wheat line nor to powdery mildew infection.

What are the possible mechanisms that cause this effect on *M. dirhodum*? The mycelium of the mildew fungus forms a fluffy white layer and covers the leave surface. This might hinder aphids from piercing the plant tissue with their stylets leading to decreased feeding performance. The pathogen infection might also cause changes in the consistency of the plant epidermis e.g. formation of thicker cell walls due to a higher evaporation rate (A. Leuchtmann, personal communication), or defence mechanisms of the plant (S. Brunner, personal communication), yet those are all speculations. Assuming such effects are more or less local, aphids could just move to a more suitable part of the leave. Also both hypotheses fail to explain why only one aphid species is affected but not the other. We thus think it is more likely that changed plant physiology is the reason.

Fungal pathogens alter the allocation of plant metabolites and induce plant defence mechanisms (27) which might affect aphid performance. Changes in the carbohydrate composition of phloem-sap could explain the different reactions of the two aphid species. It is known that sucrose is the dominant sugar compound in phloem-sap (28) and Pescod *et al.* (29) showed a species-specific response of aphids to changed sucrose levels in the phloem-sap with some aphid species having decreased population growth, whereas others remained unaffected. This could explain the species-specific reaction. To get a rough estimate about changes in plant metabolites we determined the C:N ratio of the different wheat lines in the three experiments but did not find any treatment effects. However, the C:N ratio might be too rough an estimate to detect changes in phloem sap composition and finer methods such as phloem-sap or aphid honeydew-analyses are needed to confirm compositional phloem-sap changes and their influence on the two aphid species.

Since in this study aphids were not given the possibility to choose their host plant and all aphid populations were started with ten individuals, we lead the reduced population size of *M. dirhodum* back to decreased overall fitness caused by increased development times and/or decreased fecundity on the powdery mildew infected plants. The positive density-dependence

of individual size means that big populations also consisted of bigger aphids. It has been shown that for aphids life-history traits such as individual size, development time and fecundity are all positively correlated (30) an observation we also made in a life-table experiment using *M. dirhodum* [unpublished data, based on von Burg *et al*. (31)]. We therefore think that the bigger *M. dirhodum* populations on the healthy plants are due to a cumulative effect of shorter development times and higher fecundity, all traits that were unaffected in the life-table experiment using the same Pm3b-transgenic wheat lines but without mildew infection (31). In contrast to *M. dirhodum* there was a negative relationship between body size and population size for *R. padi* but only in the Bobwhite GM/control comparison where populations were highest. Thus, the negative density-effect on aphid size is probably due to a crowding effect.

GM pathogen-resistant plants offer advantages and new options in pest management strategies promising decreased use of pesticides. Our study could clearly show that bigger aphid populations were due to an indirect effect of powdery-mildew resistance and implies that the control of one pest results in healthier plants and in turn becomes more favourable for another potential pest. Similar effects have been reported for example from insect-resistant transgenic Bt-cotton. Plants that were protected from the attack of the cotton bollworm *Helicoverpa armigera* (Lepidoptera: Noctuidae) became more attractive for mirid bugs due to increased numbers of flowers and bolls, their preferred feeding sites (32). GM plants that are protected from a particular biotic constraint thus need to be studied in respect to their susceptibility against other antagonists to ensure that they can be deployed in a sustainable way.

Materials and methods

INSECT AND PLANT MATERIAL

Laboratory cultures of *M. dirhodum* and *R. padi* were founded from individuals collected from several wheat fields around Zurich (Switzerland) during summer 2007 and 2008, respectively. The cultures were reared on the winter wheat variety Camedo and kept in climate chambers at 22°C, 80% r.h. and a 16:8 hours light:dark regime.

In total we used eight different wheat varieties. Six of them were the commercially available varieties Bobwhite, Casana, Fiorina, Frisal, Rubli and Toronit. Their seeds were provided by the Agroscope Reckenholz-Tänikon Research Station ART. In addition we worked with two experimental wheat lines, Pm3b*#1* and Chi/Glu(A13), which both had been genetically modified to carry an enhanced resistance against powdery mildew, and their respective controls. The transgenic line Pm3b*#1* was formed from Bobwhite and carries the transgene *Pm3b* of wheat which confers specific resistance to powdery mildew (24, 25). As a control we used its corresponding non-segregant sister line Sb*#1*. The Pm3b*#1* plants are further described in Zeller *et al.* (33). The second transgenic line Chi/Glu(A13) was formed from the Swiss spring wheat variety Frisal containing barley chitinase and β-1, 3-glucanase coding sequences (HvGLU) (34). This line is further described in Bieri *et al*. (35). Chitinases and glucanases are known for their anti-fungal effect increasing resistance to fungal infection in many cases (26). However, it has been shown that the resistance against powdery mildew is not increased in the Chi/Glu(A13) line (35). The seeds of the two experimental lines were provided by the Institute of Plant Biology, University of Zurich and the Institute of Plant Science, ETH Zurich.

POWDERY MILDEW STRAINS

We used two different powdery mildew strains of wheat. The first strain No.96229 is known to be avirulent on the *Pm3b* transgene (36), whereas the second strain No.98229 has been shown to be virulent on the *Pm3b* transgene (25) meaning it can break the resistance mechanism and infect plants harbouring the *Pm3b* transgene. Both mildew strains were

obtained from a collection at the Institute of Plant Biology, University of Zurich and are in the text referred to as strain A and strain V.

EXPERIMENTAL PROCEDURE

In the Variety comparison we worked with the six commercially available wheat varieties and mildew strain A. The two treatments were either inoculated with mildew or not inoculated for both aphid species resulting in a total of 240 plants (2 treatments × 2 aphid species × 6 wheat varieties × 10 replicates). In the GM/control comparison we used the two GM lines [Pm3b*#1* and Chi/Glu(A13)] and their controls (Sb*#1* and Frisal). As in the first experiment we used mildew strain A and the treatments were as well inoculated or not inoculated resulting in 128 plants (2 treatments × 2 aphid species × 4 wheat varieties × 8 replicates). In the Bobwhite GM/control comparison we only used the wheat lines Pm3b*#1* and Sb*#1* but included both mildew strains A and V. There were three treatments: not inoculated plants, plants inoculated with strain A and plants inoculated with strain V. Totally there were 120 treatment combinations (3 treatments × 2 aphid species × 2 wheat varieties × 10 replicates).

The following procedure was applied to all three experiments. The experimental plants were individually grown in single pots (3 L) under constant conditions (22°C, 60% r.h., 16:8h) in the greenhouse. We used compost soil and fertilized each pot with 3 g of Osmocote Exact slow release granulates when sowing (N15 : P9 : K9 : Mg3, Scotts Italia SRL, Italy). In addition plants were fertilized weekly with about 150 ml of a 0.2 % aqueous solution of Vegesan Standard (80g N, 70g P_2O_5, 80g K_2O, Hauert HBG Dünger AG, Grossaffoltern, Switzerland). Plants were watered as required. Three weeks after planting, the seedlings were moved into two separate greenhouse compartments (with the same conditions) where half of the plants were inoculated with powdery mildew. Inoculation of the plants was done by brushing which provides a rapid and fairly uniform inoculation method (37). Since powdery mildew is an obligate biotrophic pathogen, in vitro cultivation is not possible. The inoculum

was therefore produced by infecting the susceptible wheat variety Kanzler. These infected host plants were then equally rubbed over the experimental wheat lines. In the Bobwhite GM/control comparison, one third of the plants were inoculated with strain A, one third with strain V and the remaining third were not inoculated.

After the inoculation all plants were caged with plastic bags (Egli Plastic AG, Dällikon, Switzerland) to prevent future aphid and mildew cross-contamination and inoculated and not inoculated plants were kept separately for the next two weeks during which the mildew infection symptoms established. After these two weeks the plants were equally distributed over two greenhouse compartments so that each compartment contained the same number of replicates per treatment × wheat line × aphid species combination. Due to its size, experiment one was conducted in two temporal blocks including five replicates for each treatment combination per block.

After the incubation time and the repositioning we transferred ten 1^{st} to 2^{nd} instar nymphs of either *M. dirhodum* or *R. padi* to each plant using a sucking tube. The plastic bag cages were only shortly and individually removed. The aphids were then left to establish a population for three weeks which correspond to around three aphid generations. After three weeks the experiment was stopped. Mildew infection intensity was determined on the whole canopy using the Cobb's scale ranging from zero to nine (38). The whole plants were cut just above-ground, bagged and stored in a -80°C freezer for further analysis. Subsequently, the aphid population was counted for each plant and the hind tibia length from five randomly selected adult aphids was measured under a binocular using an ocular micrometer (Zeiss, Feldbach, Switzerland). Plant above-ground vegetative biomass was determined by drying the plants at 80°C for 24 hours and weighing the dry weight to the nearest 0.01g (Mettler Toledo, Greifensee, Switzerland). Since protein-like structures are the prime source of nitrogen compounds and to a lesser degree also of carbon compounds, and since pathogen infection can lead to changes in nitrogen tissue concentrations (39), we determined the C:N ratio of five

randomly chosen plants per wheat line and treatment but irrespective of the aphid species to get a rough estimate of changes in plant metabolites. The C:N ratio was assessed from about 3 g of dried and powdered leave material by using thermal combustion (Leco CHNS-932 Elemental Analyzer, Leco Corporation, St. Joseph, MI, USA).

STATISTICAL ANALYSES

In all three experiments the data for the two aphid species were analysed separately. The number of aphids was square-root transformed to meet model assumptions, aphid size was analyzed untransformed. For the analysis of the number of aphids we included plant biomass as covariable, whereas in the aphid size analysis we included aphid number. In all three experiments we lost some replicates due to aphid cross-contaminations. Therefore degrees of freedom do not match up with the original design. In the GM/control comparison we lost all the *M. dirhodum* replicates of one greenhouse compartment due to an infection with an entomopathogenic fungus which means that we only had four replicates for *M. dirhodum* instead of eight.

The data of the Variety comparison were analysed using a linear mixed-effects (LME) model based on *F* statistics. The model included treatment, wheat variety and their two-way interaction as fixed effects and temporal block and greenhouse compartment as random effect.

The data of the GM/control and the Bobwhite GM/control comparison were also analysed with LME looking at different a priori contrasts. For the GM/control comparison we built seven orthogonal contrasts (C1-C7, Fig. 3a). First we compared the two varieties Bobwhite and Frisal with each other (C1). Within each variety we then compared the two treatments (inoculated vs. not inoculated, C2 for Bobwhite and C3 for Frisal). Finally, within each of the four variety × treatment combinations the GM lines were compared to their corresponding controls (C4-C7). Since for *M. dirhodum* we only had data from one greenhouse compartment we used a linear model (LM) to analyse the contrasts. The first contrast of the analysis of the

Bobwhite GM/control comparison was the comparison between the three treatments not inoculated, inoculated with strain A and inoculated with strain V (Fig. 4a, C1). We then compared GM vs. non-GM plants within each treatment (Fig. 4a, C2, C3, C4).

The C:N ratio was arcsine transformed and analysed using LME. In the Variety comparison, treatment, wheat variety and their two-way interaction were fixed effects. In the GM/control comparison we had treatment, variety and GM as well as their interactions as fixed effects. In the Bobwhite GM/control comparison we included treatment and GM and their interaction as fixed effects.

All analyses were done with either of the statistical software products R (R development core team) or GenStat (VSN International Ldt.).

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FIGURE 1. Mildew infection levels

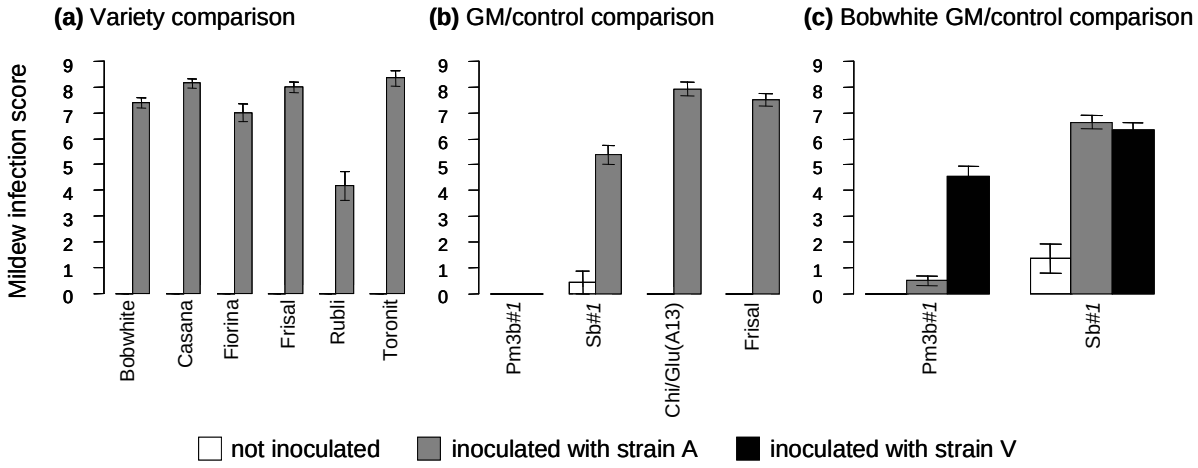

Fig. 1. Mildew infection levels (according to Cobbs' scale) of the different treatments in the three experiments (means ± s.e.m.). Open bars correspond to the not inoculated plants, grey bars stand for the plants inoculated with mildew strain A and black bars correspond to plants inoculated with mildew strain V. (a) Mildew infection of the six commercially available wheat lines of the Variety comparison. All inoculated plants were also infected. (b) Mildew infection of the two transgenic wheat lines and their corresponding control lines of the GM/control comparison. Inoculated Pm3b*#1* plants were not infected. (c) Mildew infection levels of the transgenic Pm3b*#1* line compared to the control line Sb*#1* in the three treatments of the Bobwhite GM/control comparison with the two mildew strains A and V. Pm3b*#1* plants inoculated with strain A were not infected, whereas Pm3b*#1* plants inoculated with strain V were infected.

FIGURE 2. Variety comparison

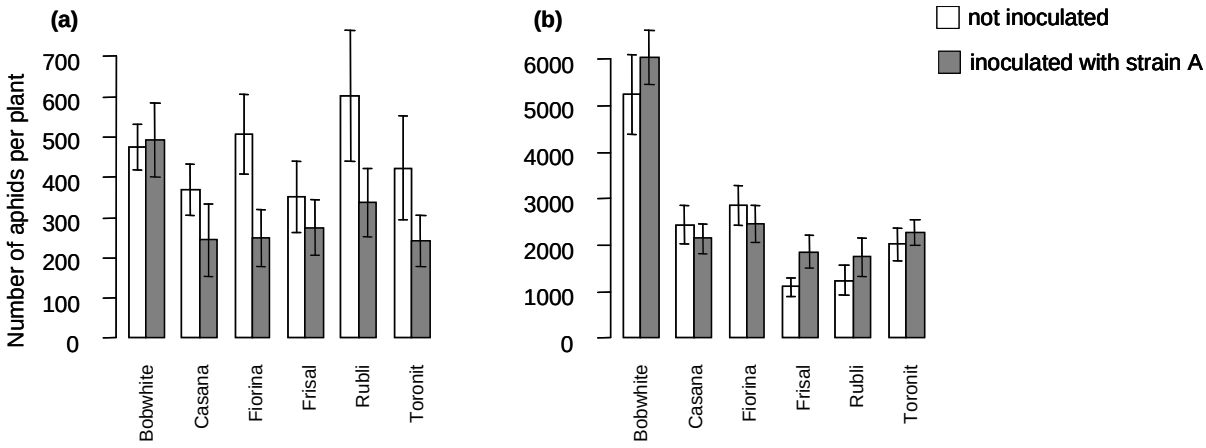

Fig. 2. Variety comparison. Aphid population sizes on the six different wheat varieties and the two different treatments of the Variety comparison (means ± s.e.m.). Open bars represent the not inoculated plants whereas grey bars represent the plants inoculated with mildew strain A. (a) Population size of *M. dirhodum*. (b) Population size of *R. padi*.

FIGURE 3. GM/control comparison

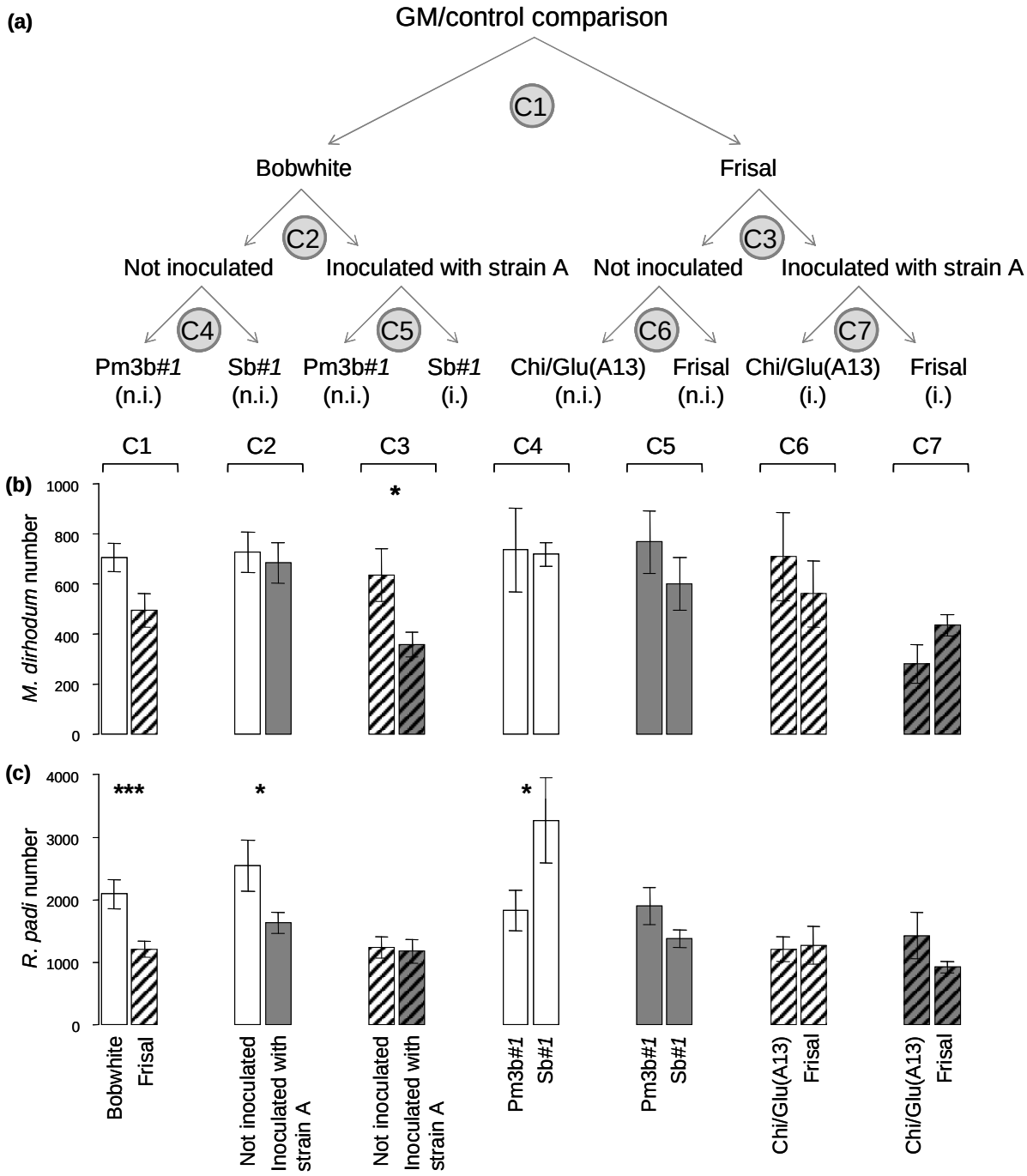

Fig. 3. GM/control comparison. (a) Description of the seven orthogonal contrasts built to analyse the GM/control comparison. The numbers in the grey circles identify the contrasts as they occur in (b) and (c). Below the names of the wheat lines in the respective treatments the infection status is given in brackets ("n.i." = not infected, "i." = infected). The barplots show the mean number (± SEM) of aphids per plant and the seven different contrasts (C1 – C7) as shown in the flow-diagram. Plain bars represent Bobwhite plants and striped bars stand for Frisal plants, whereas (except for C1) open bars represent not inoculated plants and grey bars represent plants inoculated with mildew strain A. (b) Population size of *M. dirhodum*. (c) Population size of *R. padi*. Asterisks indicate significant differences (* $p < 0.05$, *** $p < 0.001$).

FIGURE 4. Bobwhite GM/control comparison

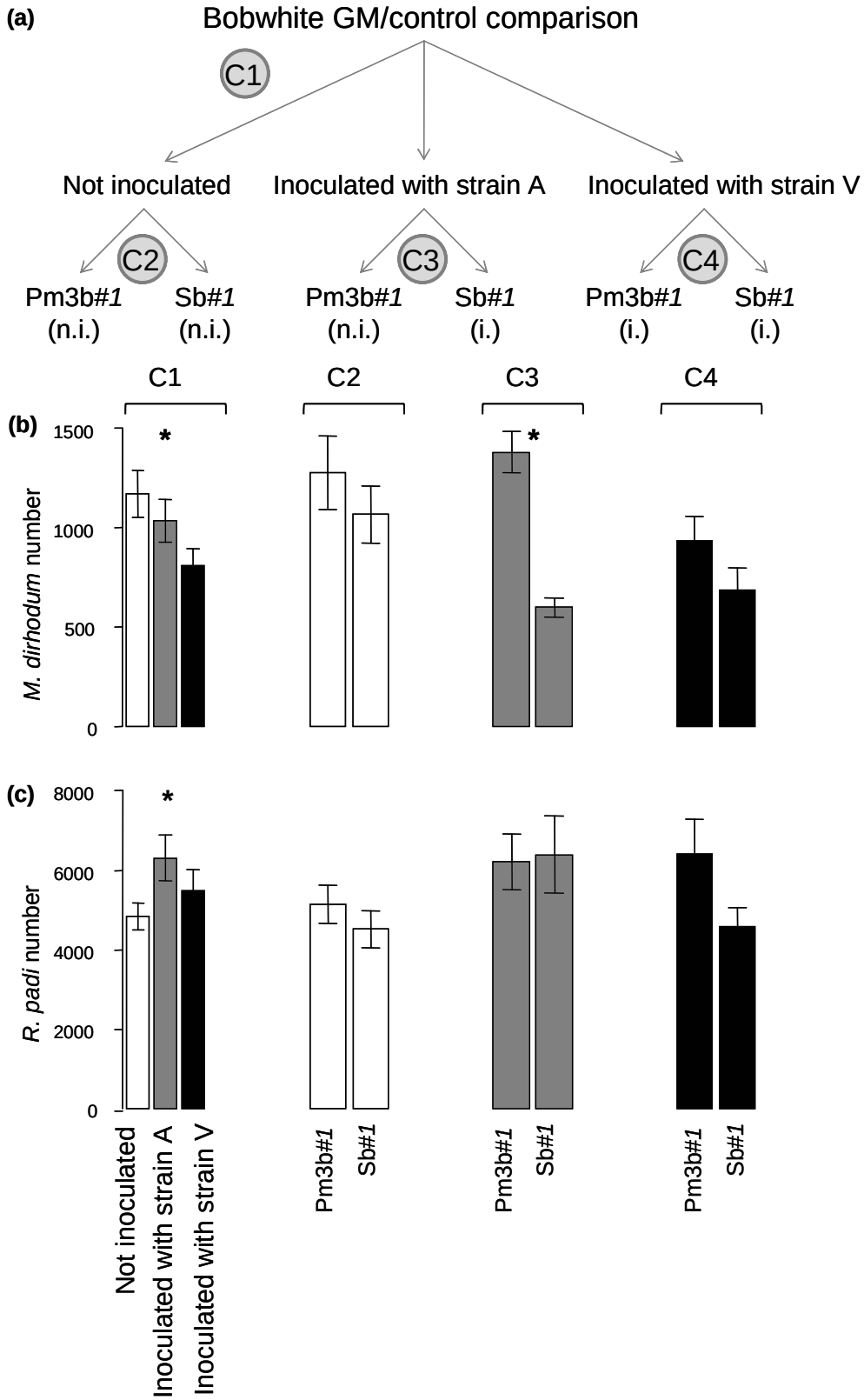

Fig. 4. Bobwhite GM/control comparison. The flow-diagram in (a) describes the orthogonal contrasts built to analyse the Bobwhite GM/control comparison. The numbers in the grey circles identify the contrast as they occur in (b) and (c). Below the names of the wheat lines in the respective treatments the infection status is given in brackets ("n.i." = not infected, "i." = infected). The barplots in (b) and (c) show the mean number (± s.e.m.) of aphids per plant and the four different contrasts (C1 – C4). Open bars represent the not inoculated plants, grey bars represent the plants inoculated with mildew strain A and black bars the plants inoculated with strain V. (b) Population size of *M. dirhodum*. (c) Population size of *R. padi*. Asterisks indicate significant differences (* $p < 0.05$, *** $p < 0.001$).

APPENDIX

Potential costs of facultative endosymbionts to the aphid *Metopolophium dirhodum* (Walker)

SIMONE VON BURG

p.93-102

Potential costs of facultative endosymbionts to the aphid *Metopolophium dirhodum* (Walker)

Simone von Burg

In Chapter 2 I described a life-table experiment performed with 30 different aphid clones (von Burg *et al.*, 2010). Like earlier studies (Dedryver *et al.*, 2001; Moran, 1991; Vorburger, 2005) I found significant clonal variation in all measured life-history traits (von Burg *et al.*, 2010). However, there is an additional factor that needs consideration when comparing aphid clones. In many cases, differences between aphid clones that have originally been attributed to genetic variation, have been found to be caused by the presence or absence of facultative endosymbiotic bacteria (Chen *et al.*, 2000; Montllor *et al.*, 2002; Oliver *et al.*, 2005; Tsuchida *et al.*, 2004). In addition to the obligate endosmybiont *Buchnera aphidicola*, which provides aphids with essential amino acids (Douglas, 1998), there is a range of facultative endosymbiotic bacteria that infect aphids. Different clone lines can possess different endosymbionts or even be doubly infected. Endosymbionts are transmitted vertically from the mother to her offspring, and the endosymbiont infection of a clone line is usually stable. That is why their presence was often neglected and their effect ascribed to genetic factors of the aphid. Only five years ago Moran *et al*. (Moran *et al.*, 2005) named three of these endosymbiotic bacteria, all belonging to the Enterobacteriaceae: *Hamiltonella defensa, Regiella insecticola* and *Serratia symbiotica*.

Shortly after their detection, endosymbiotic bacteria have been shown to improve aphid defence against parasitoids and pathogens (Oliver *et al.*, 2003; Scarborough *et al.*, 2005) or to influence aphid performance on different host plants (Tsuchida *et al.*, 2004). There are also attempts to use aphid endosymbionts in novel aphid and virus management strategies. With the use of genetically modified Indian mustard (*Brassica juncea*), Banerjee *et*

al. (2004) identified a plant lectin that binds to endsymbiont derived chaperonins in the aphid gut. The expression of this lectin leads to the reduced survival of the mustard aphid *Lipaphis erysimi* on these plants and even has the potential to inhibit virus acquisition (Banerjee *et al.*, 2004; Dutta *et al.*, 2005).

Alongside with the life-table experiment described in Chapter 2 (von Burg *et al.*, 2010) I also screened the 30 aphid clones for endosymbiotic bacteria to determine their influence on aphid performance on the eight different experimental wheat lines.

Material & Methods

During the life-table experiment I collected the offspring produced by the test individuals and stored them separately in 90 % EtOH. To detect and identify endosymbionts in the different aphid clone lines, DNA was extracted from five to ten individuals of the collected offspring using the DNeasy blood and tissue kit from Qiagen (Crawley, United Kingdom). Universal bacterial primers (10F, 35R) (Russell & Moran, 2005; Sandstrom *et al.*, 2001) were used to amplify parts of the endosymbionts' 16S ribosomal RNA gene for direct sequencing using the PCR cycling parameters described in Darby *et al*. (2001). Endosymbionts were then identified by comparing these sequences with published sequences of facultative endosymbionts in GenBank (Benson *et al.*, 2005). Whenever I inferred double infections, characterized by two overlying sequences, I confirmed the endosymbiont species present by using diagnostic PCR (Haynes *et al.*, 2003).

Data analysis

Having identified the endosymbionts of the aphids I analysed the data in GenStat (VSN International Ldt.) using analysis of variance (ANOVA) for unbalanced data. The five life-history traits were analysed depending on the endosymbiont infection which was a factor with five levels (no infection, infected with *R. insecticola*, infected with *R. insecticola* and

unknown, infected with *H. defensa*, infected with *R. insecticola* & *H. defensa*). Block, aphid morph and wheat line were included as cofactors. None of the dependent variables were transformed, as they already met the assumptions of normality and homoscedasticity of residuals, except for total number of offspring which was transformed to the power of two (y^2). Significant heterogeneities of group means found in the ANOVA were further investigated using multiple comparisons to find out which means differed from each other. I conducted a Tukey-Kramer test, which is designed for unequal sample-sizes (Tukey, 1953). The post-hoc analysis was done in R using the package DTK.

Results

As expected, endosymbiont infection was stable within the individual *M. dirhodum* clone lines and did not change with wheat line. There was only one *M. dirhodum* clone which was endosymbiont-free. I found nine clones which were singly infected with *R. insecticola* and five clones singly infected with *H. defensa*. Fifteen clones were doubly infected with both endosymbiotic bacteria. Furthermore, two clone lines were doubly infected with *R. insecticola* and an additional, unidentified bacterium (von Burg *et al.*, 2010).

The only life-history trait for which the factor endosymbiont was significant was the fitness parameter F_i' ($F_{4,25} = 2.87$, $p = 0.044$). All the other life-history traits were unaffected by the endosymbionts (Fig. 1). The multiple comparisons revealed that aphids with a single infection of *H. defensa* had a significantly lower F_i' compared to aphids without endosymbionts, aphids with *R. insecticola* and aphids doubly infected with *R. insecticola* and *H. defensa* (Fig. 1e). There was no significant difference between aphids with *H. defensa* and aphids doubly infected with *R. insecticola* and the unidentified bacterium (Fig. 1e).

Discussion

As mentioned in the introduction, endosymbiotic bacteria play a crucial role in aphid biology. I detected two known endosymbiotic bacteria *H. defensa* and *R. insecticola* and one unknown bacterium in the 30 clones of *M. dirhodum*. All except one clone line were infected with either one or two of these endosymbionts. *Hamiltonella defensa* has been shown to enhance defence against parasitoids (Oliver *et al.*, 2003) whereas *R. insecticola* increases resistance to a fungal pathogen (Ferrari *et al.*, 2004; Scarborough *et al.*, 2005) and has only recently been associated with increased parasitoid resistance (von Burg *et al.*, 2008; Vorburger *et al.*, 2010).

All these studies have shown that having endosymbionts is advantageous for aphids as they increase resistance to natural enemies or pathogens and hence, it is surprising that not all aphids carry endosymbionts. Global surveys for *Acyrthosiphon pisum* have shown that facultative endosymbionts are only found at intermediate frequencies in nature (Oliver *et al.*, 2006; Sandstrom *et al.*, 2001; Simon *et al.*, 2003; Tsuchida *et al.*, 2004). This indicates that the presence of endosymbionts might only pay off under strong parasitoid and pathogen pressure. If parasitoid or pathogen pressure is low, potential costs of having endosymbionts might exceed the benefits of their presence.

However, up to the present only one study has been able to provide evidence for such costs (Oliver *et al.*, 2008). While that study did not find any fitness costs to aphids harbouring *H. defensa* in a laboratory life-table assay in the absence of parasitoids, it showed that infection frequencies in a population experiment increased in the presence of parasitoids and decreased in their absence. If carrying endosymbionts is generally costly, then it is surprising that among my 30 *M. dirhodum* clone lines 29 carried endosymbionts and only one clone line was endosymbiont-free. In the present study I found that of the three different endosymbionts present in my clone lines one, *H. defensa*, induced costs, as interpreted from the reduced fitness of the clones carrying this endosymbiont. Single infections with this endosymbiont were only present in five out of 30 clone lines. Hence, clones carrying only this endosymbiont might have been selected against. Population studies in the presence and absence of

parasitoids or pathogens should be conducted to see how the frequency of this endosymbiont in the aphid community would change as a consequence. It is interesting that aphids doubly infected with *H. defensa* and *R. insecticola* performed better than aphids only infected with *H. defensa*. This indicates that *R. insecticola* somehow compensates for the costs induced by *H. defensa*, perhaps by reducing its abundance.

This study was not designed to detect costs of endosymbionts and the number of clones having certain endosymbionts is therefore unequal. However, I still found multiple clone lines for all the different endosymbiont-infection levels with the exception of endosymbiont-free aphids with only one clone line. Here it is difficult to determine to what extent the observed effects were caused by aphid genotype rather than the absence of endosymbionts. Experiments including curing clones from endosymbionts and infecting endosymbiont-free clones with endosymbionts could help to disentangle the two effects.

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FIGURE 1

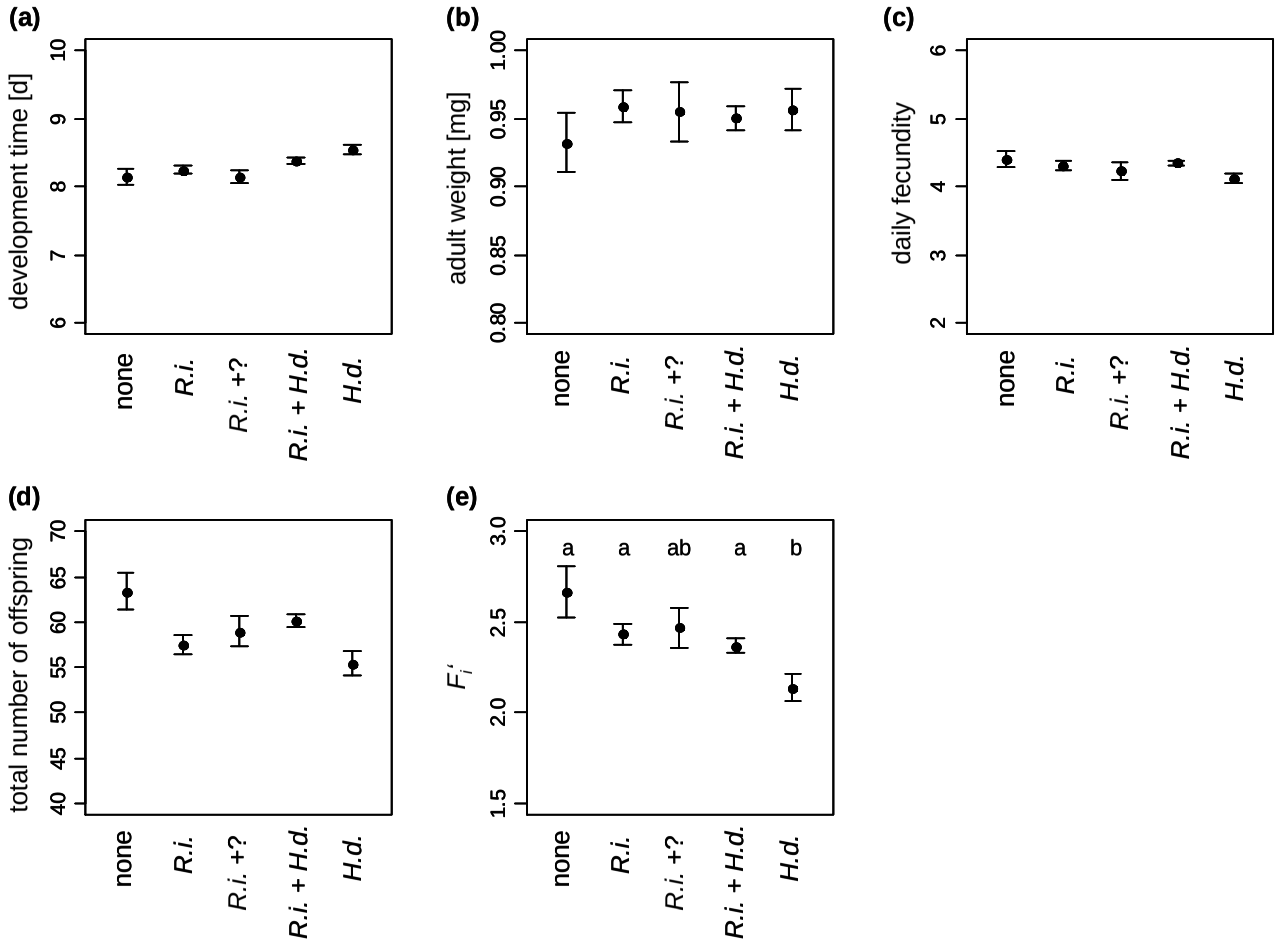

Fig. 1. The five life-history traits depending on the different endosymbiont infection levels (means ± SEM): none = no endosymbionts, *R.i.* = *Regiella insecticola*; *R.i.* +? = *Regiella insecticola* and unknown, *R.i. + H.d.* = *Regiella insecticola* and *Hamiltonella defensa*, *H.d.* = *Hamiltonella defensa*. (a) development time; (b) adult weight; (c) daily fecundity; (d) total number of offspring; (e) F_i'. We only found significant differences for the fitness estimate F_i'. The significances of the multiple comparisons are indicated with letters.

GENERAL CONCLUSIONS

In this project we investigated the effects of GM wheat plants on insect herbivores amongst which we worked with aphids and their associated food webs. We hypothesized that alterations in the metabolism of GM wheat plants could affect feeding behaviour, growth and fitness of insect herbivores and their natural enemies. We investigated our hypothesis in the field, in a semi-field environment (convertible glasshouse), and under confined conditions (glasshouse, climate chambers). The experiments in the field and the convertible glasshouse focussed on naturally occurring herbivore populations and on aphid-parasitoid food webs. Complementary experiments in the glasshouse and climate chambers were performed to better understand the mechanisms driving the plant-insect interactions.

The key findings achieved in this study are that we could not detect any major direct effects of GM plants on insect herbivores and their associated parasitoids that exceeded the natural variation between crops, wheat cultivars and/or study years. Furthermore, the differences that we did find were not consistent between study years. This is not surprising and has been observed before in numerous studies about the non-effects of Bt-transgenic crops (Sanvido *et al.*, 2007). However, we found an interesting indirect effect of the GM plants on aphid abundance: reduced mildew infection of the GM wheat lines resulted in bigger aphid populations.

The effects of GM plants on non-target organisms can be divided into two groups each of which consists of two sub-groups (Sanvido *et al.*, 2007). First, there are effects related to the transgenic product. Non-target species can either be directly affected by toxic transgenic products (e.g., Bt toxins) or indirect toxic effects can occur for example due to the fact that the nutritional quality of sensitive herbivores is affected by the toxin which has indirect consequences for a predatory species. Second, there are effects that occur independently from a transgenic product. These include unintended effects due to the genetic modification or

changes in the agricultural practice. Along with the engineering of new disease-resistant biotech crops that do not produce insect-active toxins, such as the wheat lines used in this study, these effects which occur independently from a transgenic product, might increase in importance. We provided evidence for such an unintended effect and clearly showed that increased aphid populations were due to an indirect effect of powdery-mildew resistance which implies that the control of one pest results in healthier plants and in turn becomes more favourable for another pest. Similar effects have been observed for example with insect-resistant transgenic Bt cotton. Plants that were protected from the attack of the cotton bollworm *Helicoverpa armigera* (Lepidoptera: Noctuidae) became more attractive for mirid bugs due to the increased numbers of flowers and bolls, their preferred feeding sites (Whitehouse *et al.*, 2007). Such positive non-target effects raise the question what is the lesser of two evils and GM plants that are protected from a particular biotic constraint thus need to be studied in respect to their susceptibility against other antagonists to ensure that they can be deployed in a sustainable way. Additionally, there are indirect effects due to changes in the agricultural practice. In this thesis we do not provide information about the implications on pesticide regimes especially under field conditions which needs further testing.

One of the goals of this work was to assess the suitability of aphids as indicators for non-target risk-assessment procedures. We detected only very small direct effects of the GM plants on aphid clones which raises the question whether aphids do not react so sensitively to changes in the host plant after all. However, in other experiments we showed that aphids do react to the GM plants, even though these were indirect effects through mildew infection intensity. Furthermore, we have shown that wheat varieties and crop species affect aphid performance. Hence, we are confident that we actually detected all the existing differences between GM plants and their controls and think that due to their easy cultivation in the laboratory, and to their omnipresence in agricultural ecosystems, aphids are a good model organism to assess the potential environmental impacts of GM crops.

Another goal was to test the suitability of the convertible glasshouse for future risk-assessment studies. Unfortunately, there were too few aphids and parasitoids in the field to compare the two systems statistically. Compared to the field, everything, beginning with the mildew infection levels to aphid and parasitoid abundance, was much higher in the convertible glasshouse, where protection against unfavourable weather conditions seemed to boost natural aphid and parasitoid population growth. Due to the automatic roof and side walls, plants were not exposed to strong winds or rainfalls which benefited the growth of the aphid population. We also assume that the mean seasonal temperature is higher in the convertible glasshouse compared to the field which leads to faster development and reproduction in aphids. Higher mildew infections could be due to higher humidity in the convertible glasshouse. However, even though, we could not statistically analyse the field data, we found the same aphid and parasitoid species as in the convertible glasshouse. The fact that the convertible glasshouse seems to boost natural aphid and parasitoid population growth, might actually make it easier to detect differences and might thus, be an advantage when studying the effects of GM wheat on insect herbivores.

Agricultural ecosystems per se have negative impacts on biodiversity (Ammann, 2005; Chapin *et al.*, 2000; Hails, 2002; Robinson & Sutherland, 2002; Tilman *et al.*, 2002). The intensification of agriculture, which amongst others includes the use of pesticides, has triggered a cascade of declining biodiversity, beginning with insect species up to birds (Chamberlain *et al.*, 2000; Robinson & Sutherland, 2002). In this context GM crops that reduce agricultural inputs such as pesticides might actually be beneficial in terms of biodiversity as it is for instance the case with Bt cotton. The use of Bt cotton has resulted in a significant reduction of pesticide applications (FAO, 2004; Fitt *et al.*, 2004) which in turn directly resulted in fewer non-target effects of the formerly used pesticides (Romeis *et al.*, 2006; Wolfenbarger *et al.*, 2008). It is certain, that the adoption of GM crops includes favourable and less favourable impacts on the environment as the adoption of any other

agricultural technology so far. The risks of GM crops should therefore always be put in the context of their benefits and the risks of the current agricultural practice.

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PEER-REVIEWED PUBLICATIONS

von Burg, S., Van Veen, F.J.F, Álvarez-Alfageme, F., & Romeis, J. (2010) Aphid-parasitoid community structure on genetically modified wheat. *Biology Letters*, In press

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